STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME LXIII

NEW YORK

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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WAVERLY PRESS
BALTIMORE, U. S. A.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 969–981.]

PHYSICAL, CHEMICAL, AND BIOLOGICAL STUDIES ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

COMPARISON WITH THE VIRUS OF FOOT-AND-MOUTH DISEASE.

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(Received for publication, February 2, 1927)

In an earlier paper[1] attention was drawn to the resemblances between the effects produced by the incitant of foot-and-mouth disease and of vesicular stomatitis. The characteristics to be especially mentioned are: propagation in guinea pigs in continuous series,[2] the correspondence of the experimental disease produced, and the possibility of transmitting vesicular stomatitis to cattle and swine. Such slight discrepancies as the infrequency of secondary lesions were ascribed to variability of strains of the virus. The two viruses were found to show parallel responses to selective filterability. On the other hand, certain clear-cut distinctions were noted, such as want of cross-immunity in guinea pigs, cattle, and swine, and the failure of the horse to respond to inoculation with the virus of foot-and-mouth disease, while being highly susceptible to the virus of vesicular stomatitis.

The present article deals with experiments on the physical, chemical, and biological characters of the virus of vesicular stomatitis, which may serve as a basis for wider comparison with that of foot-and-mouth disease, and at the same time provide data bearing on taxonomy. The latter subject has received, until the present, scant attention.

[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet Med. Assn.*, 1926, lxx (N S. xxiii), 147. The Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry should also be consulted.

[2] This has also been done with vesicular stomatitis virus by Cotton, W. E., *J. Am. Vet. Med. Assn.*, 1926, lxix (N.S. xxii), 313.

Description of the Strain of Virus Employed.

Source of Material.—Through the kindness of Dr. John R. Mohler, Chief of the United States Bureau of Animal Industry, and of Dr. W. E. Cotton, Assistant Superintendent of the Bureau's Experiment Station at Bethesda, Maryland, a sample of virus designated as that of vesicular stomatitis was received early in October, 1926. The specimen consisted of lingual vesicle coverings of a cow at the experiment station, which was inoculated in turn with material collected from a New Jersey cow ill with the disease. An extensive epidemic of vesicular stomatitis was raging at the time in northern New Jersey, and in the vicinity of Port Jervis, New York. The virus was active, for Dr. Cotton reproduced the disease in test horses in 20 hours. The material was sent in 50 per cent glycerol. A fragment 2 × 4 mm. was removed from the glycerol, washed in phosphate buffer at pH = 7.5,[3] (ground with sterile sand, and suspended in the buffer solution. About 0.1 cc. of this was injected by the method described[4] intradermally in the posterior pads of two guinea pigs. After 48 hours both animals showed the typical primary, or inoculation vesicles of experimental vesicular stomatitis, already described.[1] Up to the present the virus has been propagated in guinea pigs through at least 90 consecutive passages.

The clinical course of the affection in these animals was a counterpart of experimental foot-and-mouth disease. Secondary vesicles appeared in uninoculated pads in about half the animals; none was observed in the mouth or on the tongue. In these respects, we confirmed our earlier observations.[1] But the irregular occurrence of secondary lesions produced by this strain of virus does not indicate an essential difference between it and the virus of foot-and-mouth disease. We have already stated[1] that certain samples of foot-and-mouth disease virus may act in this manner in guinea pigs or in cattle.

In general, it may be stated that, apart from the likeness of the clinical course, other effects in guinea pigs of this strain of vesicular stomatitis virus are identical with those of the virus of foot-and-

[3] Phosphate buffer, as mentioned here and elsewhere in this article, is made by adding 2.5 gm. of potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

[4] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, and the Bureau Report.[1]

mouth disease.[6] For example, the mortality rate among 450 guinea pigs showing vesicular stomatitis was about 1 per cent; the affection is also practically non-lethal. The virus was free from constant, visible microorganisms, and on two occasions, when secondary, chance, microscopic bacteria were found admixed with the virus, they could be removed by Berkefeld filtration without injury to the specifically active agent. On the other hand, after inducing its specific effect, the latter invited invasion of ordinary bacteria—a character common to filter-passing viruses generally—so that a vesicle turned pustule on the 3rd to 4th day. Furthermore, in about 95 per cent of the animals the period of incubation was 18 to 48 hours, and the period became shorter and the severity of the disease increased as the concentration of the virus was augmented. Moreover, blood withdrawn 20 to 24 hours after intradermal inoculation was active. Aspirated vesicular contents showed the presence of virus in greatest concentration when this material was obtained from lesions up to 24 hours old. From this time to 72 hours a gradual diminution in virulence took place until after 3 days, when the vesicular contents, or ground infected pad tissues, were only exceptionally active. Finally, resistance of recovered animals to reinoculation with active material from the same source was marked, and the immunity lasted for at least $4\frac{1}{2}$ months.

Hence this sample of the virus of vesicular stomatitis is similar to the one already described[1] and induces in guinea pigs effects indistinguishable from those of the virus of foot-and-mouth disease.

Propagation in Rabbits.

The transfer of vesicular stomatitis to rabbits either has not been attempted, or has been unsuccessful and therefore not reported.

[6] The articles mentioned in Foot-note 12 should be consulted for a description of the effects of the virus of foot-and-mouth disease in guinea pigs. Since it is forbidden to work with the latter in the United States, no direct comparison could be made employing the same stock of normal guinea pigs, and the same laboratory conditions in the case of both viruses. For this reason no cross-immunity tests could be made here, although the results of a wide experience with another strain of the virus of vesicular stomatitis have already been reported.[1]

The rabbit appeared important for making additional tests on the above two viruses, and, incidentally, on another virus, namely, that of febrile herpes.

Comparison with Foot-and-Mouth Disease.—Gins and Fortner[6] found that rabbits could be infected with guinea pig virus by scarification of the mucous membrane of the inner lip surface. Vesicles appeared, and the contained fluid was in turn active for normal rabbits or for guinea pigs. Sixteen consecutive rabbit passages were thus effected. Nicolau and Galloway,[7] employing guinea pig virus, were able to induce local vesicles after intralingual injections. In neither case was there evidence of secondary lesions nor of generalization of the vesicular process.

We studied the susceptibility of rabbits to vesicular stomatitis by injecting the virus in the brain, cornea, and buccal mucous membranes.

The rabbits failed to show any untoward effect after intracranial injection of the active Berkefeld V filtrate obtained from guinea pig pads, which was in the ninth guinea pig passage. In another test, virus was employed which was adapted to rabbits by successful corneal inoculation. The materia of the third, fourth, and fifth corneal passages was kept in 50 per cent glycerol The glycerol was renewed weekly three times, the corneal tissue was then removed, washed, suspended in saline solution, and injected intracranially. While the injected material was active for the pads of guinea pigs, it failed to affect rabbits upon intracranial inoculation.

It appears, therefore, that the virus of vesicular stomatitis, like that of foot-and-mouth disease,[8] is non-neurotropic. Furthermore, in sharp contrast to the effects of herpetic virus, none of the guinea pigs injected with the virus of vesicular stomatitis showed microscopic evidences of damage to the nervous tissues.

On the other hand, the rabbit reacts specifically to corneal inoculations.[9] Beginning with filtered suspensions of infected pads of the

[6] Gins, H. A., and Fortner, J., *Berl. tierärztl. Woch.*, 1926, xlii, 89; *Centr. Bakt.*, *1. Abt.*, *Ref.*, 1925, lxxviii, 576.

[7] Nicolau, S., and Galloway, I.-A., *Compt. rend. Soc. biol.*, 1925, xciii, 1283.

[8] For the non-neurotropic effects of foot-and-mouth disease virus consult Levaditi, C., Alberta-Lorente, R., and Galloway, I., *Compt. rend. Soc. biol.*, 1926, xcv, 387.

[9] For mode of inoculation see Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

guinea pig, and later employing the ground corneal scrapings, all of thirteen rabbits, during five consecutive passages, showed typical vesicles along the lines of incision of the cocainized cornea. The lesions appeared from 2 to 4 days after application of the virus and were accompanied by conjunctival inflammation. The vesicles coalesced as a rule, and in two rabbits left small, localized opacities. There was no fever at any time. After 7 days healing began, so that within 2 weeks the eye returned to normal. 10 days later the recovered corneæ were refractory to reinoculation. As little or no work was done in this way with foot-and-mouth disease virus, no comparisons can be made.

As is the case with foot-and-mouth disease virus, scarification of the inner surface of the lips and cheek of the rabbit, and application of filtered suspensions of infected pads of guinea pigs led, within 24 to 48 hours, to distinct, localized vesicles. The histopathology of the lesion is similar to that of the guinea pig pads from experimental vesicular stomatitis and foot-and-mouth disease.

The rabbit, therefore, is susceptible to the virus of vesicular stomatitis, and where comparisons can be made with that of foot-and-mouth disease in the same animal, its effects appear to be identical. The rabbit and the guinea pig are epitheliotropic, but not neurotropic toward the viruses.

Comparison with Herpes.—A limited number of experiments was made with the H. F. strain of herpetic virus described by Flexner and Amoss.[9] This virus, injected intracranially in a rabbit, produced the typical cerebral symptoms and death in 4 days. A portion of the base of the brain was suspended in saline solution equal to a 10 per cent suspension, and of this 0.1 cc. was injected intradermally in the posterior pads of three guinea pigs. In general, the effects were similar to those described by Gildemeister and Herzberg.[10] Edema of the pads was noted, and this persisted for about 4 days, when vesicles appeared. These were often pustular from the start, coalesced, tended to necroses, and lasted for about a week. In addition the frequent occurrence of gangrene in the phalangeal and metatarsal areas, and in all of ten guinea pigs injected through three consecutive passages, retention of urine and feces, and paralyses of the posterior extremities were present. The nervous symptoms first appeared about 5 to 6 days after injection. Only one of the ten animals survived. No secondary vesicles were observed.

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[10] Gildemeister, E., and Herzberg, K., *Deutsch. med. Woch.*, 1925, li, 97.

These comparisons show the herpetic virus to be distinct from the viruses of vesicular stomatitis and of foot-and-mouth disease. These differences are emphasized by the fact that guinea pigs recovered from experimental vesicular stomatitis are susceptible to the herpetic virus and *vice versa*.

The reactions of rabbits to corneal inoculation of the herpetic and of the vesicular stomatitis virus differ. In the former the affection induced is much more severe locally, and encephalitic signs may become evident; in the latter, the lesions in the eye are ordinarily mild, tend to complete and rapid recovery, and no nervous manifestations are observed. Cross-immunity is absent in the recovered animals.

Histopathology of Experimental Vesicular Stomatitis of the Guinea Pig.

In pads of guinea pigs inoculated intradermally with filtered or unfiltered material containing the virus of vesicular stomatitis, the first changes noted are swelling and thickening of the epidermis and derma, as a result of edema and cellular infiltration. The infiltrating cells consist mainly of polymorphonuclear neutrophils (the so called pseudo-eosinophils) and less numerous, monocytes (or macrophages or endothelial leucocytes). After 18 to 24 hours vesicles appear between the epithelial layer and corium, and between the horny and Malpighian strata. These layers are filled with serum at first, but soon cells, especially the neutrophils, invade the fluid. In the corium, however, the vesicles may be filled with blood, with an eventual greater cellular infiltration. At this stage occur active mitosis and striking intranuclear changes, to be described immediately. After 48 to 72 hours, necrosis of epithelial cells is observed and retrogression begins; the vesicles are filled with multinucleated cells and later with granular material and droplets of various sizes. They now begin to contract and to dry. After 3 to 4 days, the horny layer exfoliates, a large number of neutrophils is present, and with this a proliferation of epithelial cells about the site of the vesicle. Thereafter healing begins and the lesion is covered with a scab composed of leucocytes and epithelial cells. After the 8th day, as a rule, the cells of the epidermis appear normal for the most part, and the vesicles are replaced by a highly vascularized granulation tissue.

The nuclear changes of the epithelial cells in the Malpighian layer,

in infiltrating cells of the corium and of the vesicles consist in a condensation of the chromatin about the wall, thus leaving only a shadow of nuclear structure which is surrounded by a darker staining, denser membrane. The nucleoli and nucleolar degenerated particles are stained bluish by Giemsa's or the eosin-methylene blue methods. Within the membrane may be seen, in specimens stained by Giemsa's method, one or more perfectly rounded, light pinkish staining bodies, about 1.5 microns in diameter. Some are smaller, but rarely are there any larger. These changes are most marked in lesions 24 hours old, and are comparable to the so called inclusion bodies described by Gins[11] as characteristic of foot-and-mouth disease. In lesions which are 48 hours old, a large number of somewhat different bodies are noted. They are round or oval and vary in diameter from 2 microns to a size large enough to fill almost the entire nucleus with the exception of a narrow, clear zone between the body and nuclear membrane. With Giemsa's or eosin-methylene blue stains, they appear pink to red in contradistinction to the blue nuclear membrane, and, as a rule, lie on a clear background. Some nuclei may contain from two to four of the smaller sized bodies. In general, they are similar to but not necessarily identical with the intranuclear inclusions which characterize certain other filter-passing viruses.

To summarize, the histopathology of experimental vesicular stomatitis is identical with that of experimental foot-and-mouth disease, described by several observers, notably by Gins,[11] and by Levaditi and his coworkers.[8] Furthermore, we have found indistinguishable pathological conditions in foot-and-mouth disease, not only of guinea pigs, but also of cattle and swine. Finally, it is important to note that the virus of vesicular stomatitis can be classified as one of a group of ultra-microscopic agents, the effects of which are characterized by the presence of peculiar intranuclear changes. Further studies by Dr. Rivers on their significance are in progress

Titration of Virus.[12]

The virus of vesicular stomatitis, as it exists in ground infected guinea pig pads, or in aspirated vesicular contents, could be diluted 1:10,000,000, but not higher.

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[11] Gins, H. A., *Centr. Bakt., 1. Abt., Orig.*, 1922, lxxxviii, 265.

[12] In this, as in other experiments, the technical procedures of experiments are omitted. For details of methods, see Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, 685, 815, 833, and the Bureau Report.[1]

and still show activity. In filtered material a 1:10,000,000 dilution also induced the experimental disease in guinea pigs. In the blood of this animal, withdrawn 20 to 24 hours after pad inoculation, the virus was present in much lower concentration: the limit of infectiousness being 1:2000 to 1:200,000.

According to these tests which show the limit of infectiousness at 1:10,000,000 in filtered or unfiltered material, the virus of vesicular stomatitis corresponds in activity to that of foot-and-mouth disease.[4]

Failure of Sedimentation of the Virus.

Centrifugation, at 3700 revolutions a minute for 2 hours, of material containing the virus of vesicular stomatitis failed to bring about its sedimentation. In graded dilutions of 1:30 to 1:300,000, the topmost and the lowest layers behaved in all instances alike. In this respect also the two viruses are similar.[4]

Selective Filtration through Chamberland Bougies.

In an earlier paper[1] it was stated that the behavior of the virus of vesicular stomatitis on filtration paralleled that of the virus of foot-and-mouth disease. Both passed readily through Seitz' asbestos discs and Berkefeld V candles, but not always through Berkefeld N filters The same was true in the case of Chamberland bougies, both viruses passing through the L 3 and L 7, but usually not through the L 11 type [13] Hence it was concluded that the two viruses had the same tendency to adsorption in the walls of denser, electronegative filters

Because of the importance of the L 11 bougie in differential filtration, the tests have been repeated with the particular sample of vesicular stomatitis virus at hand. Seven trials were made with the active material suspended in phosphate buffer at pH = 7.5 and at 8.5. At pH = 7.5, the virus passed through only one of three bougies but a portion of the same active material at pH = 8.5 traversed all of the four L 11 filters employed.

In the case of the virus of foot-and-mouth disease, a similar phenomenon was interpreted as evidence that the incitant is electropositive—a conclusion which was confirmed by the behavior of the virus on cataphoresis and by the determination of its isoelectric range at pH = about 8. Although cataphoresis tests have not been

[13] Olitsky, P. K., and Boez, L., *J. Exp. Med.*, 1927, xlv, 685.

made with the virus of vesicular stomatitis, the indications are that it conforms with that of foot-and-mouth disease in respect to magnitude, charge, and isoelectric range.

Effect of Hydrogen Ion Concentration on Viability.

It has already been shown by Bedson and Maitland,[14] by Stockman and Minett,[15] and by us[16] that the virus of foot-and-mouth disease survives longest in a medium of which the hydrogen ion concentration is at pH = 7.5 to 7.6, and that viability is diminished considerably by slight variations above or below this narrow range. The same conditions apply to the virus of vesicular stomatitis. For example, the latter remained alive in phosphate buffer at pH = 7.2 and 7.5 to 7.6 for at least 52 hours at 37°C., but was inactive at pH = 6.8 and 8.0 at this time. After 100 hours, however, only the material at pH = 7.5 to 7.6 was active. The precise end-point was not determined.

Resistance to Chemicals.

The virus of foot-and-mouth disease is highly resistant to chemicals, such as alcohol, ether, chloroform, glycerol, and to many so called antiseptics, such as bichloride of mercury, cresol, phenol, etc.[14,15,17] We have pointed out that this remarkable resistance is due to the fact that the chemicals coagulate the proteins of the medium in which the virus is suspended and these in turn prevent direct contact of the virus with the reagents. If the periodic phenomenon attending such processes is considered and coagulation is prevented, the virus is made to come directly under the influence of the chemicals. Under these conditions, it is more sensitive to destruction by them than is staphylococcus. Moreover, the virus of foot-and-mouth disease is destroyed as rapidly as staphylococcus, or even more rapidly, by substances

[14] Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[15] Stockman, S., and Minett, F. C., *J. Comp. Path and Therap.*, 1926, xxxix, 1.

[16] Olitsky, P. K., and Boez, L., *J. Exp. Med.*, 1927, xlv, 833, and the Bureau Report.[1]

[17] Olitsky, P. K., and Boëz, L , *J. Exp. Med.*, 1927, xlv, 815, and the Bureau Report.[1]

such as sodium hydroxide (1 to 2 per cent) or antiformin (1 per cent), which do not form coagula.

A similar series of tests was made with the virus of vesicular stomatitis in which we selected as an example of the narcotic solvents, 60 per cent alcohol, and of other coagulating substances, bichloride of mercury (1:1000), cresol (3 per cent), and phenol (1 per cent). Of the non-coagulating chemicals sodium hydroxide (2 per cent) was chosen.[18]

The virus was still active after 24 hours in 60 per cent alcohol. But if sodium hydroxide (1:5000), in which it can survive for at least a day, was added, and coagulation thus prevented,[17] it was killed within 1 minute. In bichloride of mercury, cresol, and phenol, in the dilutions mentioned, active materials remained viable for at least 6 hours. Tests for longer periods were not made. In sodium hydroxide (2 per cent) the virus was killed within 1 minute. An additional test revealed that 3 per cent cresol containing 1 per cent sodium hydroxide also inactivated it within 1 minute.

The virus remained active for at least 4½ months in 50 per cent glycerol buffered at pH = 7.5, and kept at 4–6°C.

In so far as the resistance to chemicals is concerned, therefore, the virus of vesicular stomatitis resembles that of foot-and-mouth disease.[17]

Survival of the Virus Outside the Body.

It has been found[18] that in moist or palpably dried garden soil, the virus of foot-and-mouth disease survives for at least 25 days, and in hay for at least 1 month. Active materials derived from vesicular stomatitis also maintain their activity for a considerable time after leaving the body. Infected guinea pig vesicle coverings remained infectious for at least 31 days in garden soil kept at 4–6°C., or at 20°C., in a moist or in a palpably dried state.

Respiration of the Virus.

A study of the respiration of the virus of vesicular stomatitis was undertaken with the idea of obtaining information concerning its living character. The active agent could not, however, be separated in a pure state from respiring living tissues, nor could it maintain its

[18] Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry.

life under the conditions imposed by the experiment. Such was also the case with the virus of foot-and-mouth disease.[18]

Effect of Ultra-Violet Light on Viability.

In contrast to the results of the tests on respiration are those of the following experiments, done with the collaboration of Dr. F. L. Gates, of The Rockefeller Institute, on the effects of different wave-lengths and energies of monochromatic ultra-violet light. Since no similar tests were made with the virus of foot-and-mouth disease, no comparison of the two viruses can be offered in this respect. The experiments are reported, however, as adding suggestive evidence on the relation of the incitant of vesicular stomatitis to living bacteria. This evidence was obtained by comparing the behavior of the virus with that of *Staphylococcus aureus* under similar exposure to ultra-violet irradiation.[19]

Aspirated vesicular contents diluted 1:10 in buffered broth at pH = 7 4 were employed as virus and a thin suspension of *Staphylococcus aureus* from an 18 hour broth culture as control. The surface of thin layers of 2 per cent agar buffered at pH = 7.4 in small Petri dishes was washed with each of these materials respectively. After exposure, the virus-agar was cut within the limits of the area of light penetration, ground in a mortar, suspended in phosphate buffer at pH = 7.5, and of this about 0.4 cc. was injected intradermally into the posterior pads of guinea pigs. An additional control of unexposed virus-agar strips of similar size was also used in each test. After exposure, the agar, seeded with staphylococci, was incubated overnight at 37°C., and the number of colonies appearing in the exposed areas was compared with that in like areas from the unexposed portion of the plates.

At lambda = 2675 Ångström units, with a total energy of from 512 to 540 ergs per sq. mm., all the staphylococci were killed; at the same wave-length and from 256 to 270 ergs per sq. mm., 87 to 97 per cent of the organisms were killed. In respect to the virus subjected to the greater energy all of five guinea pigs were negative after inoculation, and in the second instance in which the lesser energy was used only one of four guinea pigs showed the experimental disease. All of five guinea pigs injected with unexposed virus-agar (controls) revealed typical lesions.

At lambda = 3022 Ångström units, with a total energy of 23,300 to 29,900 ergs per sq. mm., 97 to 100 per cent of the staphylooocci were killed. The exposed

[19] Olitsky, P. K., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 431.

virus-agar inoculated into four guinea pigs failed to infect, but the unexposed virus-agar (control) induced the experimental disease in all of four animals.

At lambda = 3126 Ångstrom units with a total energy of 60,400 ergs per sq. mm., there was no visible effect on the staphylococci. The exposed virus-agar proved active in three of four guinea pigs, and the unexposed similar material, as a control, induced the experimental disease in all of four animals.

It is thus evident that the transmissibility of the virus of vesicular stomatitis is lost on exposure to the same wave-lengths and energies of monochromatic ultra-violet light that are bactericidal. Furthermore, at the limits of destructive action of ultra-violet light, the reaction of the virus parallels that of the microorganism. Since adsorption of specific energies is one index of chemical character, these parallel reactions suggest that the substance of the virus is similar in character and chemical constitution to bacterial protoplasm.

DISCUSSION.

In an earlier paper[1] reference was made to the similarity of the virus of vesicular stomatitis and of foot-and-mouth disease. Slight differences between them were ascribed to the variability of different strains. In the foregoing pages, additional evidence is presented to show their resemblance in several other reactions, physical, chemical, and biological. Furthermore, the clinical appearance of the diseases produced by the two viruses may be the same. Under field conditions and among cattle, the method employed heretofore in the United States for differential diagnosis has been to inject suspected material into a horse. If it reacted with typical vesicular lesions, a diagnosis of vesicular stomatitis was made; if it did not, the material was designated as having been derived from foot-and-mouth disease.[20]

There are, therefore, under consideration two viruses differing practically only in an absence of cross-immunity in recovered animals and in the resistance of the horse to one of them. In view of the non-cultivability of either virus, it is difficult to prove the precise relationship of one to the other. Yet, if a comparison be made with what exists among the types of foot-and-mouth disease virus itself or among known, cultivable bacteria, a close relationship between the two may be inferred. For example, there are at least two types of

[20] Mohler, J. R., *U. S. Dept. Agric., Dept. Circular 400*, 1926.

the active agent of foot-and-mouth disease, either of which shows no cross-immunity to the other in recovered animals. While all strains are generally active in cloven foot animals, there are some to which the guinea pig is resistant. Here, then, is a genus containing types which do not exhibit cross-immunity and show different pathogenic effects in a different species of animal. Among examples of similar behavior of cultivable microorganisms may be mentioned those dealt with in the recent studies of Tillett.[21] The rabbit which is susceptible to infection with Type I and Type II pneumococcus, is practically resistant to Type III. It is well known, moreover, that these different types do not show cross-immunity.

It appears, therefore, from the resemblance of the viruses of vesicular stomatitis and of foot-and-mouth disease, as demonstrated in this and in an earlier paper,[1] that their relationship is close. But this is an inference based on indirect evidence and is tentative until artificial cultivation of the viruses is obtained.

CONCLUSIONS.

A taxonomic study of the virus of vesicular stomatitis is presented along with evidence additional to that already reported[1] to show the similarity of this virus to that of foot-and-mouth disease. The connection of the two is discussed and the deduction drawn that their generic relationship is close. On the contrary, the differences between these two viruses and the herpetic are sufficiently marked to indicate a lack of generic connection among the three.

The results of a comparative study on the effects of particular wave-lengths and energies of monochromatic ultra-violet light on the virus and on *Staphylococcus aureus* reveal that the adsorption of specific energies by the two is parallel. Since the adsorption of specific energies is an index of chemical character, these experiments suggest that the virus is similar in character and chemical constitution to bacterial protoplasm.

[21] Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 1093.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 1045–1056.]

ON THE INFLUENCE OF ACID GROUPS ON THE SEROLOGICAL SPECIFICITY OF AZOPROTEINS.*

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PLATE 31.

(Received for publication, January 20, 1927.)

In previous work (2–5) investigations were made of the relation between chemical constitution and serological specificity by compounding various substances of simple chemical structure with proteins and using the resulting combinations as antigens. The coupling of proteins with diazonium derivatives of aromatic substances offered itself as the most suitable means. The immune sera obtained were tested by the precipitin reaction. In order to avoid species-specific reactions on the protein component the test antigens generally were prepared with a protein from another species than that employed for the immunizing antigens. The principal result of these studies was the observation that the immune sera exhibit a specificity which is determined by the chemical structure of the simple compounds attached to the protein.[1]

Aside from the phases already dwelt upon there are others to which the method outlined is applicable. The present paper deals with the influence of acid groups on specificity. Reference has been made to this question in one of the papers quoted (3).[2] For our purpose antigens were prepared with chemicals with and without acid groups; after immunization with these substances the immune sera were tested with both types of antigens.[3]

* Twenty-second paper on antigens and specificity. *Cf.* Reference 1.

[1] For a fuller review we refer to "The chemical aspects of immunity," by H. Gideon Wells, American Chemical Society Monograph Series, New York, 1925. page 77.

[2] Page 387.

[3] For the sake of brevity these will be referred to in the following as acid and non-acid antigens and immune sera respectively.

EXPERIMENTAL.

Preparation of the Antigens for Immunization.—As an example the method used for aniline is given. The other substances were taken in quantities equivalent to that of the aniline

1.2 gm. of aniline were dissolved in 10 cc. of water and 5 cc. of 7 N HCl and diazotized with the necessary amount of sodium nitrite at a temperature of 0–5°C., with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 100 cc. and poured into a mixture of 100 cc. of horse serum and 100 cc. of normal sodium carbonate. The solution must give a strongly alkaline reaction with phenolphthalein. Coupling was allowed to take place for 10 minutes at 0–5°C. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was suspended in a small amount of water. On addition of some normal sodium hydroxide and vigorous stirring it became viscous or jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the material.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol it was brought into solution with alkali as before and after dilution it was reprecipitated with hydrochloric acid and filtered. After grinding in a mortar the substance was dissolved in water at alkaline reaction. Finally the reaction was adjusted with hydrochloric acid to faint alkalinity or neutrality

The volume was made up to 190 cc. with distilled water and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 10 cc. of a 5 per cent phenol solution were added. The presence of some suspended azoprotein in the solution does not affect its use for the immunization.

The other preparations were made in a similar way Some of them such as the azoproteins from ortho- and para-chloroaniline are still less soluble, therefore a great part of the substance remains in suspension. The diazotization of para-nitroaniline was carried out by adding at room temperature the sodium nitrite solution to a suspension of the finely powdered substance containing the required amount of hydrochloric acid. Small amounts of undissolved material were removed by filtration. This same method was applied to ortho-nitroaniline and 1, 2, 5-nitrotoluidine which were only used for the preparation of test antigens. In the case of the amino acids[4] as para-arsanilic acid, para-aminobenzoic acid, etc., the azoproteins can be dissolved without difficulty. For coupling diazotized ortho-aminobenzoic acid with horse serum, 50 cc. of normal NaOH per 100 cc. serum were used instead of sodium carbonate.

Immunization.—Eight rabbits were injected intraperitoneally with 15 cc. or less of each antigen at weekly intervals. At least two immune sera of sufficient strength were obtained after three to six injections in every case. Only one serum was obtained in the instance of para-chloroaniline.

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[4] For further details see Reference 3, pages 354–364.

Antigens for the Tests.—The azoproteins for the test solutions were prepared with chicken serum in place of horse serum in the manner described above. The diazo solution (in a few instances containing undissolved diazo compound) was added to a mixture of serum and sodium hydroxide (1 part of normal sodium hydroxide to 2 parts of serum) and the coupling allowed to take place at room temperature for half an hour. After precipitation with dilute hydrochloric acid the azoprotein was filtered and washed with water.

There was some difficulty in that the preparations did not yield clear solutions or were only sparingly soluble. This was overcome by treatment of the azoprotein with alkali. An amount of the antigen corresponding to 5 cc. serum was ground in a mortar, made up to 10 cc. with water and 10 cc. of normal NaOH was added. After half an hour the mixture was neutralized with 10 cc. normal HCl, the precipitated azoprotein centrifuged and taken up in about 25 cc. saline containing 0.5 cc. normal NaOH. After a few minutes hydrochloric acid was added until the solution was only weakly alkaline to litmus paper and the volume made up to 30 cc. The solution to which 0.5 per cent phenol had been added was clarified by intense centrifugalization and filtration through kieselguhr paper.[5]

To make the tests comparable also the antigens with acid azo components were treated with alkali in the same manner (concerning their preparation see Reference 3, pages 362 and 363) To obtain solutions of antigens made from the halogen substituted anilines it was necessary to prolong the treatment with alkali. A quantity of the azoprotein corresponding to 5 cc. serum was ground and 20 cc. of normal NaOH added. The mixture was shaken with beads for 2 hours, neutralized and the precipitated azoprotein was taken up in 25 cc of saline containing 0.5 cc. normal NaOH. After addition of hydrochloric acid until the solution was weakly alkaline to litmus it was brought up to 30 cc., centrifuged and filtered through kieselguhr paper.

The quantity of antigen present in the solutions was determined by precipitating the azoprotein with alcohol, drying and weighing.

The solutions were found to contain from about 20 to 45 mg. of dry material in 5 cc.

Tests.—The antigens were diluted to 1:100 of a 1 per cent solution and 0 2 cc. of this solution was used for the tests. The tests were kept at room temperature and were in some instances also read after standing overnight in the ice box. The intensity of the reactions is indicated as follows. 0, f tr (faint trace), tr. (trace), ±, +, + ±, etc.

Tests with Azoproteins Made from Aniline and Various Substituted Anilines.

Tables I and II illustrate the action of various immune sera on the azoproteins prepared from aniline and a number of substituted anilines.

[5] Macherey, Nagel and Co., Düren, Germany.

TABLE I.

Of the immune sera 779 and 788 four drops were used, and of the others 2 drops. The readings were made after 1 hour at room temperature.[6]

Azoproteins prepared from chicken serum and:	Immune sera obtained from antigens made from horse serum and:									
	Aniline	Aniline	Ortho-chloroaniline	Ortho-chloroaniline	Para-toluidine	Para-toluidine	Para-toluidine	Para-nitroaniline	Para-nitroaniline	Para-chloroaniline
	No 760	No. 761	No 788	No 793	No. 763	No 766	No. 770	No. 775	No 795	No 779
Aniline	++±	+±	+±	++	+±	+±	++	+	±	+
o-Toluidine	++	±	++	++±	+±	+	+	±	f.tr.	±
o-Anisidine	+	0	+±	++	±	tr.	0	0	0	0
o-Nitroaniline	+	0	+	+	tr.	tr.	0	±	tr.	0
o-Chloroaniline	+±	±	++	++±	+	+	+	±	+	tr.
m-Toluidine	++	+	+±	++	+±	+	+±	+	+	+
m-Nitroaniline	+	tr.	+	+	+	±	+	+±	+±	±
m-Chloroaniline	++	+	+	+±	+±	+	+±	+	+	+
m-Bromoaniline	++	+	+	++	+±	+	+	+	+	+
p-Toluidine	+±	+	+	+	++	+±	++±	+	+	++
p-Anisidine	++	±	±	+	++±	+±	++±	+	+±	+±
p-Nitroaniline	+	±	±	tr.	+±	+	+±	++	++	++
p-Chloroaniline	++±	+	±	+	++	+±	++±	+±	++	++
p-Bromoaniline	++	+	+	+±	++	++	++±	+±	++	++
p-Iodoaniline	+±	+	+	+	++	+±	++±	+±	+±	++

TABLE II.

1,2,4-Nitrotoluidine	++	±	+	++	+±	+±	++	+±	+±	+
1,4,2-Nitrotoluidine	++	±	+±	+∓	++	+	+±	+±	+±	+
1,2,5-Nitrotoluidine	+	tr.	±	tr.	+	tr.	±	+±	+±	±
1,3,4-Xylidine	+±	±	+±	++	++	+±	++	+	+	+±
1,4,5-Xylidine	+±	tr.	+±	++	+	+	+	+	+	tr.
1,4,2-Xylidine	+±	tr.	+±	++	+	+	+	+	±	tr.
Acetyl-para-phenylene-diamine	0	0	0	0	0	0	0	0	0	0
Para-aminoacetophenone	tr.	0	0	0	+	tr.	±	+	±	0
Monomethyl-para-phenylenediamine	+±	+				+±	++	+±		

[6] The sera and the antigens are indicated by the names of the substances used in their preparation.

The experiments summarized in Table I show that the immune sera precipitate nearly all antigens. Also in most cases of negative reactions distinct precipitation occurred after standing overnight in the ice box and the weak reactions increased considerably in strength. Nevertheless a certain degree of specificity is to be observed.

The homologous reaction is always one of the strongest. The nature of the substituent is generally of but little influence; in the tests with

TABLE III.

4 drops were used of immune serum 729 and 2 drops of the others. The readings were made after 1½ hours at room temperature.

Immune sera obtained with antigens made from horse serum and	Numbers of the immune sera	Azoproteins made from chicken serum and.										
		Para-aminobenzoic acid	Meta-aminobenzoic acid	Ortho-aminobenzoic acid	Para-arsanilic acid	Sulfanilic acid	Ortho-cinnamic acid	Aniline	Para-nitroaniline	Ortho-nitroaniline	Para-toluidine	Meta-toluidine
Para-amino-benzoic acid.	816	++±	±	0	0	0	0	0	0	0	0	0
Para-amino-benzoic acid .	818	+±	0	0	0	0	0	0	0	0	0	f.tr.
Ortho-amino-benzoic acid .	729	0	0	++	0	0	0	±	±	±		±
Para-arsanilic acid	722	0	0	0	++++	0	0	0	0	0		f.tr.
Aniline	760	0	0	0	0	0	0	++±	+	+	+±	++
Para-nitro-aniline	775	0	0	0	0	0	0	+	+±	+±	+	+
Para-toluidine..	770	0	0	0	0	0	0	++	+±	0	++±	+±

several immune sera however the intensity of the reactions was more diminished by the presence in the azo component of a nitro or a methoxy group than by the other substituents tested.

More pronounced is the effect produced by the position of the substituents regardless of their nature. In the tests with immune sera against para substituted azoproteins the strength of the reactions decreases in a general way in the order para, meta, ortho. This sequence is reversed in the reactions with sera against the ortho sub-

stituted aniline. Observations similar to these have been made previously (3, 5). The aniline immune sera seem to react somewhat more weakly with ortho substituted antigens than with the others.

No definite regularities appear from the tests of Table II except for the negative or weak reactions with the antigens prepared from acetyl-para-phenylenediamine and para-aminoacetophenone. The other antigens behave similarly to those included in Table I.

Tests with Azoproteins Prepared from Amino Acids.

A considerable number of immune sera prepared with acid azoproteins were described previously (3).

TABLE IV.

2 drops of aniline immune serum No. 760 were used. Readings after 1 hour.

Azoproteins made from chicken serum and the following substances	Antigen dilutions 1 to			
	100	300	1000	3000
Aniline	+++	++±	+	±
Ortho-nitroaniline	+	+	tr.	0
Meta-nitroaniline	++	+±	±	tr.
Para-nitroaniline	+±	+±	±	tr.
Ortho-anisidine	+	+	±	tr.
Para-anisidine	+±	+±	±	f tr
Para-aminobenzoic acid	tr.	0	0	0
Sulfanilic acid	tr.	0	0	0

The present experiments as recorded in Table III demonstrate the considerable influence of acid groups on the specificity.

Table IV gives the results of tests made with various concentrations of the antigens.

The sera prepared with "non-acid" antigens give no precipitation with the "acid" azoproteins.[7] In the converse experiment showing the action of the "acid" immune sera on the "non-acid" antigens, there were weak positive reactions especially with one of the four sera and

[7] Distinct precipitation occurs in higher concentration of the acid antigens. These reactions were shown to be non-specific flocculations since such reactions took place also with entirely unrelated immune sera as precipitins for rat, pig serum, etc.

some more weak reactions came up after keeping the tests overnight. The reactions with serum No. 729 increased somewhat in strength.

A representative experiment is illustrated by Figs. 1 and 2.

In conformity with the findings already referred to (3) the sera for acid antigens exhibit in general a considerably higher specificity than the others. This is further substantiated by a series of tests in which the antigens for the reaction *in vitro* were made with the same protein as that used for the immunization (horse serum). Also in this way the difference between the two sorts of antigens is brought out clearly (Table V; *cf.* Fig. 3) although the method would tend to destroy the specificity of the reactions, due to the fact that the immune sera react to a certain degree upon the protein part of the antigens.

TABLE V.

To 0.2 cc. of the diluted antigen 1 drop of immune serum was added. The first reading was taken after 5, the second after 15 minutes.

Immune sera obtained with antigens made from horse serum and·	Numbers of the immune sera	Antigens made from horse serum and										Horse serum	
		Aniline		Para-toluidine		Para-nitroaniline		Para-arsanilic acid		Para-amino-benzoic acid			
Aniline...	760	++±	+++	+±	++	++	+++	tr.	±	0	tr.	±	+
Para-toluidine .	763	++	++	++	++±	++	++	tr	tr.	0	0	+	+
Para-nitro-aniline	735	+±	++±	+	++	++	+++	tr.	±	0	±	tr.	±

Reactions with an Azoprotein Made from the Methyl Ester of Para-Aminobenzoic Acid.

In order to prove in a different way the results obtained the following experiments were carried out. Starting from the ester of an aromatic amino acid an azoprotein was prepared. Presumably this should react with the non-acid immune sera like the other non-acid antigens. If this azoprotein is treated in such a way as to bring about hydrolysis of the ester it should no longer react with such sera but should have acquired by virtue of the free carboxyl group the property of reacting with an immune serum specific for the corresponding acid.

The methyl ester of para-aminobenzoic acid was prepared according to the directions given by Einhorn (6). The recrystallized substance melted at 112°C.

Diazotization.—0.190 gm. of the finely powdered substance was suspended in 10 cc. of water. Normal hydrochloric acid and a 2 per cent solution of sodium nitrite were added alternately in small quantities keeping the solution distinctly acid to Congo red and adding the nitrite after a test with potassium starch iodide paper showed the disappearance of nitrous acid. The theoretical quantity of nitrite was used up. The solution was kept at 0–5°C. This diazo solution was added to a mixture of 10 cc. of chicken serum and about 7 cc. of normal sodium carbonate, cooled to 0–5°C. The mixture was distinctly alkaline to phenolphthalein but an excess of alkali was avoided to prevent hydrolysis of the ester. After coupling for 10 minutes at 0–5°C., and acidifying with hydrochloric the azoprotein was filtered and washed with water. It was made up with saline into a fine suspension (volume 20 cc.) and made slighly alkaline to litmus by addition of 0.1 cc. of normal sodium hydroxide. Undissolved material was removed by centrifuging. The azoprotein was precipitated by neutralization with hydrochloric acid and after centrifugalization it was redissolved in 10 cc. of saline with alkali, carefully avoiding an excess. The solution which was very faintly alkaline to litmus was centrifuged and filtered through kieselguhr paper. Its content of azoprotein was estimated in the manner described before. The test solution was made up to 1/100th of a 1 per cent solution.

The experiment turned out as was anticipated. The solution of the ester-azoprotein was precipitated by aniline and paratoluidine immune sera and not by an immune serum for para-aminobenzoic acid except for a weak reaction observed when the tests were kept overnight in the ice box (Table VI). This is in agreement with the behavior of other non-acid azoproteins.

Hydrolysis of the Ester-Azoprotein.—To 2 cc. of a 1 per cent solution of ester-azoprotein were added 2 cc. of 1/10 normal sodium hydroxide and samples of 0.5 cc. were taken from the mixture at various times, *i.e.*, immediately after mixing, and after being kept 5 minutes, 1, 4, 6, 8 and 20 hours, at room temperature. These samples were adjusted to faint alkalinity with 1/10 normal hydrochloric acid, the azoprotein thus being kept in solution.

Table VII gives the reactions with the two immune sera at various stages of the hydrolysis.

TABLE VI.

2 drops of immune serum were used. The antigen concentration corresponded in all cases to 1/100 of a 1 per cent solution. Readings after 1 hour and after standing overnight in the ice box.

Azoproteins made from chicken serum and	Immune sera obtained from antigens made from horse serum and							
	Aniline		Para-toluidine		Para-nitroaniline		Para-aminobenzoic acid	
	No 760		No. 770		No 775		No 816	
	1 hr.	Night	1 hr.	Night	1 hr	Night	1 hr	Night
Aniline	++	++±	++	++±	+	++	0	tr.
Para-toluidine	+±	++	++	++±	+	++	0	tr.
Para-nitroaniline	+±	++	+±	++	+±	+++	0	±
Para-aminobenzoic acid	0	0	0	0	0	0	++	++++
Para-aminobenzoic ester	+	++	+	++	±	++	0	±

TABLE VII.

2 drops of immune serum were used. Readings after 1 hour at room temperature and after standing overnight in the ice box.

Azoproteins prepared with chicken serum.	Immune sera obtained with antigens prepared from horse serum and:			
	Para-aminobenzoic acid		Para-toluidine	
	No 816		No. 770	
	1 hr.	Night	1 hr.	Night
Para-aminobenzoic ester azoprotein	0	tr.	+±	++
Same immediately after mixing with alkali	f.tr.	±	+±	++
Same after 5 min. hydrolysis	±	+	+±	++
Same after 1 hr. hydrolysis	+±	+±	+	+±
Same after 4 hrs. hydrolysis	+±	++	tr.	+
Same after 6 hrs. hydrolysis	++	++±	0	+
Same after 8 hrs. hydrolysis	++	+++	0	tr.
Same after 20 hrs. hydrolysis	++	+++±	0	tr.
Para-aminobenzoic acid azoprotein	++	+++±	0	0
Para-toluidine azoprotein	0	tr.	++	++±
Aniline azoprotein	0	f.tr.	++	++±

The experiment shows that during the course of the hydrolysis the precipitation with the serum for para-toluidine decreases gradually while correspondingly an increase in the strength of the reactions takes

place with the immune sera for para-aminobenzoic acid. The former reaction did not disappear completely but a trace of precipitate was still noticed after 20 hours hydrolysis when the tests were read on the following day.

SUMMARY.

The method of partial synthesis of antigens as employed in the foregoing experiments obviously cannot be substituted for the chemical study of natural antigens. But some questions of a rather general nature not easily accessible to investigations of the latter sort, may be approached by the use of artificial protein compounds. Thus the results reported indicate a peculiarity of certain chemical structures such as acid radicals.

The group of immune sera obtained by injecting azoproteins made from non-acid azo components had a wide range of activity. Substituents like CH_3, OCH_3, NO_2, Cl, Br, I, in the aromatic nucleus altered the reactions to a moderate degree only, in most cases.[8] The effects were dependent more on the position than on the nature of the substituents. Two substances were found, however, which had a pronounced effect on the specificity of the compound protein, namely acetyl-para-phenylenediamine and para-aminoacetophenone. In consideration of the above facts it is uncertain whether the antigenic changes noticed by Obermayer and Pick (7) after treating proteins with nitric acid, nitrous acid or iodine are mainly due to the substitution of hydrogen in the benzene ring by NO_2 and I, as is the general belief, or to other changes of the protein. This question had been raised already by the observation that the proteins treated with HNO_3 or HNO_2 containing respectively the nitro or the diazo group, did not differ substantially in their serological properties (8).

The antigens made from acid compounds form a group with distinctive features. In the first place the presence of acid radicals destroys the reactivity with the immune sera for the non-acid substances. This influence is so marked that even the reaction with the species-specific part of the protein, if such is present, appears to be diminished. Also the sera produced with the acid antigens react but feebly with the non-acid azoproteins. Accordingly it was pos-

[8] *Cf.* Wells,[1] page 79

sible to show that by hydrolysis of the ester of an aromatic acid contained in an azoprotein the serological reactions of the antigen underwent a radical change.

The presence of a free carboxyl group in the antigens not only determines the characteristics mentioned but there is reason to believe that it increases markedly the degree of specificity exhibited by the antigens and the corresponding immune sera, when cross-tests are made with a number of acid azoproteins and their antisera. This is brought out by a comparison of the results of the present investigation with those described previously (3). It is of interest in this respect that the specific carbohydrates found by Avery and Heidelberger in pneumococci and pneumobacilli are mostly, if not in all cases, compounds of distinctly acid character.

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EXPLANATION OF PLATE 31.

FIG. 1. Reactions of immune serum for para-toluidine No 770 with various azoproteins prepared from chicken serum and the following substances, readings after 1 hour:

1. Aniline.	7. 1, 3, 4-Xylidine.
2. Para-toluidine.	8. 1, 2, 4-Nitrotoluidine.
3. Ortho-chloroaniline.	9. Para-arsanilic acid.
4. Ortho-nitroaniline.	10. Para-aminobenzoic acid.
5. Meta-nitroaniline.	11. Sulfanilic acid.
6. Para-nitroaniline.	12. Saline control.

FIG. 2. Reactions of immune serum for para-aminobenzoic acid No 816 with various azoproteins prepared from chicken serum and the following substances, readings after 1½ hours:

1. Aniline.	3. Ortho-nitroaniline.
2. Para-toluidine.	4. Para-nitroaniline.

5. Ortho-anisidine.
6. Para-arsanilic acid.
7. Ortho-aminobenzoic acid.
8. Meta-aminobenzoic acid.
9. Para-aminobenzoic acid.
10 Sulfanilic acid.
11. Ortho-aminocinnamic acid.
12. Saline control.

FIG. 3. Reactions of immune serum for aniline No. 760 with various azoproteins and with unchanged horse serum, reading after 15 minutes

1 Azoprotein from chicken serum and aniline.
2. Azoprotein from horse serum and aniline
3. Azoprotein from horse serum and para-toluidine
4. Azoprotein from horse serum and para-nitroaniline.
5. Azoprotein from horse serum and para-aminobenzoic acid.
6. Azoprotein from horse serum and para-arsanilic acid.
7. Unchanged horse serum.
8. Saline control.

Fig 1

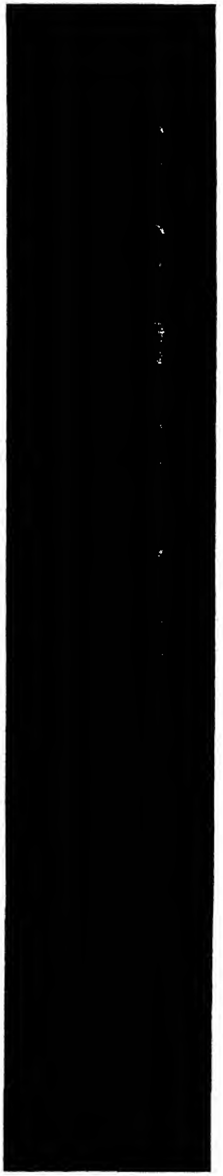

Fig. 2.

Fig 3

(Landsteiner and van der Scheer: Serological specificity of azoproteins.)

[Reprinted from The Journal of Experimental Medicine, July 1, 1927, Vol. xlvi, No. 1, pp. 197–204.]

IMMUNIZATION EXPERIMENTS WITH LECITHIN.

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(Received for publication, March 30, 1927.)

In the case of Forssman's heterogenetic antigen it was found possible to incite the production of antibodies by the injection of its specific component along with proteins. In a similar manner immune bodies were obtained with alcoholic extracts of blood corpuscles (1) and of organs (2). Since it is assumed generally though not proven that the specific part of the heterogenetic antigen is of lipoid nature it was natural to test the immunizing properties of lipoids of known chemical constitution. This was undertaken by Sachs and Klopstock (3) with lecithin and cholesterol. Indeed these authors state that they obtained antibodies by injecting into rabbits emulsions of lecithin, or cholesterol, containing pig serum. The tests were carried out mainly by means of complement fixation. A confirmatory paper was published by Ornstein (4) who also reported on successful immunizations with cephalin and with cerebrosides.

There are some points in the observations of Sachs and Klopstock not easily understood on the basis of their assumptions. Their antilecithin serum reacted on lecithin Merck but it also reacted on cholesterol and even more intensely on the latter than on a certain lecithin preparation of higher purity than lecithin Merck. This phenomenon is ascribed by Sachs and Klopstock to the presence of some cholesterol in the injected antigen but against such a view may be pointed out that in the experiments of these authors it was rather difficult to produce sera which react upon cholesterol, by injections of this substance. Another difficulty arises from the fact that the purer of the two lecithin preparations employed was the less active. The explanations for these peculiarities offered by Sachs and Klopstock do not settle conclusively the questions at issue. Since the production of antibodies for well known lipoids would be of great

significance, it seemed desirable to us to repeat the experiments with various lecithins prepared by ourselves.

EXPERIMENTAL.

We injected three lots of five rabbits each, with ox brain lecithin, egg lecithin and hydro egg lecithin, respectively. In addition sera were prepared with a commercial egg lecithin preparation (Merck) as used by Sachs and Klopstock.

Lecithin Preparations.—Lecithin No. 1. Alcoholic extract of egg yolk was treated with a 25 per cent solution of cadmium chloride in methyl alcohol, the precipitate extracted twice with ether and decomposed with methyl alcohol containing 25 per cent of ammonia. The solution was concentrated and the residue was extracted with cold alcohol. The lecithin was again precipitated as a cadmium salt and the latter was repeatedly washed with ether. The cadmium salt was then treated with methyl alcohol containing ammonia gas; the solution was concentrated, taken up in a minimum quantity of ether and precipitated with acetone.

Analysis: C 64.6; H 10.49; N 2.17; P 3 96; amino N 0.

Lecithin No. 2. 15.0 gm. of lecithin No 1 were dissolved in methyl alcohol, acetone was added until a sample on cooling to −5°C. showed the formation of a precipitate. The entire solution was then brought to a temperature of −5°C and the precipitate formed was removed by centrifugalization. The mother liquor was concentrated nearly to dryness, the residue was taken up in ether and the solution was poured into an excess of acetone. The yield was 11.0 gm. This material was dissolved in 20 0 cc. of methyl alcohol, 20 0 cc. of water were added and the solution was adjusted to pH 4. Acetone was added so long as a precipitate formed. The yield was 9.0 gm.

Analysis: C 65.3; H 10.61; N 2.00; P 4 12.

Egg Lecithin No. 3. The cadmium salt, prepared as No. 1, was extracted with ether 8 times. The lecithin obtained in this manner was further purified as follows: 26.0 gm. of lecithin were dissolved in 40 0 cc. of ether, 40 0 cc. of 10 per cent acetic acid were added, the mixture was shaken for 1 hour and the lecithin precipitated with 500 cc. of acetone.

Analysis: C 66 00; H 10.59; N 2 03; P 3.90; amino N 0.

Brain Lecithin. The cadmium salt was decomposed as usual, the filtrate was concentrated, and taken up in ether; acetone was added until a small precipitate formed. This was removed by filtration. The filtrate was concentrated nearly to dryness, the residue was taken up in ether, acetone was added to the solution to incipient opalescence. The solution was chilled to approximately −8°C. A precipitate formed which was removed by centrifugalization. The mother liquor was concentrated almost to dryness and taken up in a little ether. Acetone was added to opalescence and the solution brought to about −20°C. A precipitate formed, which was separated by centrifugalization. It was then extracted with

acetone and dried under diminished pressure. It was preserved in an atmosphere of nitrogen gas.

Hydrolecithin. This was prepared from egg lecithin by reduction with hydrogen and colloidal palladium as a catalyst.

Analysis of Merck lecithin: C 65.41; H 10.55; N 1.81; P 3.41; NH_2-N 0.

Tests were made also with a number of other samples of lecithin prepared in the laboratory with various modifications of the above described methods.

Immunization.—Rabbits were selected the sera of which gave no reactions in flocculation and complement fixation tests with emulsions of cholesterolized alcoholic beef heart extract[1] and of Merck's egg lecithin 240 mg. of lecithin were emulsified with 15 cc. of saline and 3 cc. of pig serum diluted to 15 cc. with saline were added. This emulsion was kept at room temperature for 1 hour before injection.

The rabbits received intravenous injections of 5 cc. of the lecithin emulsion generally at intervals of 3 to 5, sometimes 7 days. The sera were tested several times during the course of the experiments, with both the lecithin used for injection and Merck's egg lecithin.

Tests. The complement fixation tests with the egg, brain and hydrolecithins were carried out as follows: To 0.25 cc. of progressively doubled dilutions of the inactivated serum starting with a dilution 1:5 were added 0.25 cc. of an emulsion of lecithin (prepared by fairly rapid addition of 24 cc of saline to 1 cc. of a ½ per cent solution of lecithin in alcohol) and 0 25 cc. of guinea pig serum diluted 1:10. After incubation at 37°C. for 1 hour 0.25 cc. of sheep blood immune serum (2½–3 units) and 1 drop of a 50 per cent sheep blood suspension were added.

For the flocculation tests 1 part of a ½ per cent alcoholic solution of the egg lecithin was emulsified by fairly rapid addition of 5 parts of saline. 0.2 cc. of the emulsion was added to 0.2 cc. of the inactivated serum diluted 1:2 and the readings were taken after 20 hours standing at room temperature. For the flocculation tests with the brain lecithin and hydrolecithin a different procedure was adopted since emulsions prepared by the method described for egg lecithin were very unstable and were flocculated by most normal rabbit sera. 1 part of a ½ per cent alcoholic solution of brain lecithin was added rapidly to 5 parts of distilled water and 0.2 cc. of this liquid was mixed with 0.2 cc. of the inactivated serum diluted twice with a 2.7 per cent salt solution. In the case of the hydrolecithin 1 part of a ½ per cent solution of the substance was added by drops to 5 parts of boiling distilled water. The emulsion was filtered hot. For the test the serum was diluted with distilled water instead of saline.

The strength of the reactions in the tests is indicated as follows: Complement fixation tests—0 = no hemolysis, tr = trace, w = weak, d = distinct, str = strong, vstr = very strong, ac = almost complete, c = complete hemolysis. Flocculation tests—0 = no flocculation, ftr = faint trace, tr = trace; ±, +, ++, +++, etc.

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[1] For the technique see: *J. Exp. Med.*, 1927, xlv, 467.

By immunizing with egg lecithin Merck we obtained four strongly and two weakly active sera among six rabbits after six injections.

The sera obtained with brain lecithin and hydrolecithin gave no distinct flocculation or complement fixation with these preparations

TABLE I.

Number of sera	Injections made with	Flocculation with emulsions of: Merck egg lecithin	Merck egg lecithin freed from cholesterol	Lecithin No. 1	Lecithin No. 1 with addition of 12 per cent cholestorol
966	Egg lecithin No. 1	0	0	0	tr
967	" " " 1	+±	0	0	+
968	" " " 1	0	0	0	tr
969	" " " 1	0	0	0	0
970	" " " 1	0	0	0	0
810	Merck's egg lecithin	+++	++±	0	±
Normal rabbit No. 1		0	0	0	tr
Normal rabbit No. 2		0	0	0	±

TABLE II.

Numbers of sera	Injections made with	Complement fixation with an emulsion of egg lecithin No 1	Complement fixation with an emulsion of Merck egg lecithin
966	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c
967	" " " 1	c,c,c,c,c	0,tr,c,c,c
968	" " " 1	c,c,c,c,c	c,c,c,c,c
969	" " " 1	c,c,c,c,c	c,c,c,c,c
970	" " " 1	c,c,c,c,c	c,c,c,c,c
809	Merck's egg lecithin	c,c,c,c,c	0,0,0,0,ac,c
810	" " "	vstr,ac,c,c,c	0,0,0,0,0,ac,c
Normal rabbit		c,c,c,c,c	c,c,c,c,c

or with Merck's egg lecithin after twelve injections. Occasionally weak flocculations were noticed but after further injections the reactions disappeared.

The tests with the sera taken after twelve injections of egg lecithin No. 1 are presented in Tables I and II. Complement fixation tests

TABLE III.

Emulsions used for the tests:

A. 24 cc. of saline were added fairly rapidly to 1 cc. of a ½ per cent alcoholic solution of egg lecithin No 1. This preparation gave no Liebermann reaction for cholesterol.

B. This emulsion was made as A, with 1 cc. of a ½ per cent alcoholic solution of egg lecithin No. 1 to which had been added 0.06 cc. of a 0.25 per cent alcoholic solution of cholesterol, corresponding to 3 per cent of the weight of lecithin.

C. Emulsion made as in A, with 1 cc of egg lecithin No. 1 to which had been added 0 06 cc. of a 1 per cent alcoholic solution of cholesterol, corresponding to 12 per cent of the weight of lecithin.

D. As A, with Merck's egg lecithin. This preparation was found by the Liebermann test to contain 1 5 per cent of cholesterol or less.

E. As A, with Merck's egg lecithin from which the cholesterol was removed by dissolving 2 gm. in 15 cc. of ether and reprecipitation with 30 cc. of acetone. This purification was repeated twice. The product gave no Liebermann reaction.

F. 1 cc. of a ½ per cent alcoholic solution of cholesterol was slowly added to 24 cc. of boiling distilled water and the emulsion was filtered hot (method of Keeser (5)) The dilution of the sera was made with 1.8 per cent salt solution.

Number of sera	Injections made with	Complement fixation with emulsions of.					
		Egg lecithin No. 1	Egg lecithin No. 1 + cholesterol	Egg lecithin No 1 + cholesterol	Merck's egg lecithin	Merck's egg lecithin after removal of cholesterol	Cholesterol
		A	B	C	D	E	F
967	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c	ac,c,c,c,c	0,0,ac,c,c	0,0,ac,c,c	0,0,d,ac,c
809	Merck's egg lecithin	c,c,c,c,c	c,c,c,c,c	str,c,c,c,c,c	0,0,0,0,str,c	0,0,0,w,c,c	0,0,0,0,d,ac,c
810	" "	vstr,ac,c,c,c	ac,c,c,c,c	d,vstr,c,c,c,c	0,0,0,0,0,str,ac	0,0,0,0,w,c,c	0,0,0,0,0,str,ac
Normal rabbit No. 1		c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
Normal rabbit No. 2		c,c,c,c,c	ac,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

performed after five injections were negative as well as flocculation tests at various other times.

The serum No. 967 which gave moderate reactions, as can be seen from Tables I and II, was tested against emulsions differing in their cholesterol content in comparison with sera prepared with Merck's egg lecithin (Table III).

The highly active sera resulting from the injection with lecithin Merck gave uniformly negative tests by the method of complement

TABLE IV.

Immune sera prepared by injections with Merck's egg lecithin	Complement fixations with emulsions prepared by addition of 24 parts of saline to a 1/2 per cent alcoholic solution of			
	Merck's egg lecithin	Egg lecithin No 1	Egg lecithin No 2	Egg lecithin No 3
No. 809	0,0,0,0,d,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 810	0,0,0,0,0,tr,c	vstr,c,c,c,c	ac,ac,c,c,c	ac,c,c,c,c
" 811	0,0,0,0,ac,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 812	0,0,ac,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

Immune sera prepared by injections with Merck's egg lecithin	Flocculations of emulsions made by addition of 5 parts of saline to 1 part of a 1/2 per cent alcoholic solution of			
	Merck's egg lecithin	Egg lecithin No 1	Egg lecithin No 2	Egg lecithin No 3
No. 809	+++	±	0	0
" 810	+++	tr	ftr	ftr
" 811	++±	±	tr	0
" 812	+±	+	0	tr
Saline control	0	0	0	0

fixation with our egg lecithin preparations 1, 2 and 3. In the flocculation tests preparations 2 and 3 reacted faintly, No. 1 somewhat better, but considerably weaker than lecithin Merck (Table IV). The results with brain lecithin and hydrolecithin were similar. Some other of our preparations gave more distinct flocculation with the Merck lecithin immune sera but practically negative complement fixation as far as they were examined.

SUMMARY.

In testing several egg lecithin preparations prepared by ourselves it was found that they did not react in complement fixation tests with immune sera made by injections with commercial egg lecithin Merck. With the flocculation method two of the preparations reacted only faintly. Also the brain lecithin and hydrolecithin gave no distinct reactions.

The immunization experiments of Sachs and Klopstock could easily be confirmed when commercial egg lecithin Merck was used for the injections. Immunizations with brain lecithin and hydrolecithin yielded no active sera. With an egg lecithin (No. 1) prepared by us the results were not satisfactory though a great number of injections was made. Only one serum gave reactions of medium strength by complement fixation and in flocculation tests with emulsions of Merck lecithin. It did not react however with the lecithin preparation No. 1 itself. In this respect the results resemble to a certain degree those of Sachs and Klopstock with their lecithin Böhringer immune serum. While the reactions of Merck lecithin were slightly diminished by the removal of cholesterol, addition of cholesterol to the lecithin No. 1 had no marked effect on the complement fixation tests, even when a larger amount was added than that present in the Merck preparation. The cholesterol content of this lecithin therefore does not suffice to account for the difference in the results. It is noteworthy that our lecithin immune serum No 967 gave complement fixation with emulsions of cholesterol although this substance was not present in the injected material.

There are several plausible explanations for our results. According to one, the production of antibodies for lecithin would depend on certain physicochemical conditions of the emulsion injected or upon the presence of auxiliary substances in the lecithin preparation (*cf.* Sachs and Klopstock). Another possibility is that the active agent inducing the formation of antibodies is not lecithin itself but some other substance present in the active lecithin preparations. With regard to the latter assumption it may be mentioned that we obtained definite immunization effects from several injections of quantities

as little as 0.2 mg. of purified preparations of Forssman's heterogenetic haptene mixed with pig serum.[2]

To decide between the alternative explanations, further studies are necessary.

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[2] It may be stated that while in these experiments small quantities (1 mg.) of certain purified preparations of Forssman's heterogenetic haptene were active, no or a very slight effect was obtained on using larger amounts such as 100 mg. for each injection. These experiments will be fully described in a later communication.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1927, Vol. xlvi, No. 2, pp. 213–221.]

ON A SPECIFIC SUBSTANCE OF THE CHOLERA VIBRIO.*

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(Received for publication, April 25, 1927.)

Rather extensive work has been devoted to the question of lipoid antigens in bacilli of the acid-fast group but there are also some reports concerning the solubility in organic solvents of antigens of other microorganisms. Among these are contributions dealing with *V. choleræ.*

Levaditi and Mutermilch (1) were able to prepare solutions of cholera antigen by mixing one volume of an extract in isotonic salt solution with five volumes of absolute alcohol. For the experiments the fluid was centrifuged and the supernatant was evaporated The residue was found to contain active antigen when tested with anticholera serum in complement fixation tests. After 2 to 3 injections of 40 mg. each of the substance into rabbits the sera of the animals contained specific agglutinins, bactericidal substances, opsonins, and complement-binding antibodies. Guinea pigs treated with the substance acquired active immunity. The substance was insoluble in ether, acetone, or hot absolute alcohol. It resisted boiling in a water solution, and was not easily destroyed by dilute acid or alkali.

Prausnitz (2) in attempting to verify the findings of Levaditi and Mutermilch ascribed the effects observed by these authors to their method of filtration through paper which would not suffice to prevent the passage of bacilli into the filtrate. As a matter of fact after filtration through candles, the author was unable to demonstrate the presence of antigens in the extracts either by immunization or by tests *in vitro.*

In view of the divergent results recorded we undertook a renewed study of the subject.

EXPERIMENTAL

In order to determine the most suitable method for extraction cholera vibrios were treated successively with ether at room temperature for 24 hours and with various concentrations of alcohol by boiling under reflux for 1 hour. 10 cc. of solvent

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* See the preliminary report in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 248.

was employed for the 24 hour growth of each Blake bottle. The alcoholic solutions were filtered by means of a hot water funnel, evaporated on the steam bath to a small volume, the residue taken up in saline, and brought to a volume of 5 cc. per bottle. The solutions were put through common filter paper or kieselguhr paper (Macherey) and precipitin tests were made with the filtrates and cholera immune serum. There was no reaction with the extracts made with ether or strong alcohol but the extracts made with dilute (75 per cent) alcohol were found to be active.

Accordingly for the further work the following technic was adopted. The harvest of a 24 hour growth of cholera vibrio of 150 one quart Blake bottles was washed off with 1.5 liters saline. The microbes were centrifuged, washed twice with about 1–1.5 liters of saline and once with the same quantity of 50 per cent alcohol, centrifuging each time for 30 minutes at high speed.

The sediment was put into 95 per cent alcohol. After 1 or several days the centrifuged bacterial mass was boiled under reflux for 1 hour in 1.5 liters of absolute alcohol and filtered hot. The vibrios were then treated with boiling 75 per cent alcohol. At first two such extractions were made and the solutions joined. Subsequently the first and second extracts were kept separately. The first extraction was made with 500 cc. for 30 minutes; the second with 1500 cc for 1 to 2 hours. The extracts were filtered through a hot water funnel Because of the slow rate of filtration the filter paper had to be renewed several times. The hot filtrate ran through clear, but became turbid on cooling and when kept in the ice chest, a flocculent precipitate settled out.[1] This was separated by spinning in a cooled centrifuge and washed with some absolute alcohol and dry ether. From 150 Blake bottles the yield of the first and second extracts approximated 60 mg. and 300 mg. respectively

It seems, according to preliminary experiments, that a better yield can be obtained by isolating the substance from water extracts.

In the manner described a substance was obtained in the form of a white to grayish white powder. In water the substance swells, and slowly a viscous, more or less turbid fluid is formed; it is more readily soluble on addition of a trace of alkali. The substance was precipitated by cholera immune sera up to dilutions of 1:500,000. In higher concentrations heavy flakes were formed. Both the substances from the first and second extractions in a 1 per cent solution gave positive biuret, xanthoprotein, and Millon reactions and precipitation with trichloracetic acid, tannic acid, phosphotungstic acid, and sulfo-

[1] With another strain of *V. choleræ* and some other vibrios, the alcoholic extracts became faintly turbid on standing in the ice chest and flocculation occurred only after addition of a small quantity of salt solution.

salicylic acid. The reactions were considerably weaker with the second extract. Both preparations gave a strong Molisch test. On heating a 2 per cent solution in N/2 HCl for 90 minutes on the steam bath the liquid became turbid and a rather voluminous precipitate separated. The yield of this precipitate was about 175 mg. per gm. of the hydrolyzed substance. The supernatant fluid gave strong reduction with Fehling's solution and with phenylhydrazine an osazone crystallizing in needles, no pentose reaction with orcinol.

After 10 hours heating the content in reducing sugar was found to be 20.5 per cent, calculated as glucose. After oxidation with nitric acid the solution gave an intense reaction for phosphoric acid.

The precipitate appearing on hydrolysis showed acid character in that it was soluble in alkali, and could be precipitated from the solution by acid. It could be separated by means of methyl alcohol in two parts, one soluble in methyl alcohol and in ether, and another insoluble in these solvents. The former gave the values (calculated for ash-free substance): C, 67.03; H, 10.28; N, 1.35: the latter C, 55.83; H, 8.52; N not determined.

For testing the antigenic properties, the dry substance obtained by alcoholic extraction of the vibrios was dissolved in saline and injected into rabbits. After 2 to 3 injections of 2.0 mg., and also 0.2 mg., precipitins were formed for the extracted substance as well as agglutinins for *V. choleræ*. The antigenic activity of still smaller quantities was not tested.

The substance proved to be toxic and there was loss of animals in the immunization experiments. Guinea pigs died after intravenous injection of 1.0 mg. of the substance and sometimes even 0 1 to 0.2 mg. was lethal.

In order to exclude the presence of bacilli in the injected material immunization experiments were also carried out with 75 per cent alcoholic extracts filtered hot through Berkefeld candles tested for impermeability to a broth culture of *V. choleræ*. The precipitate settling after cooling was employed in quantities of 2 mg. per injection. The results were essentially identical with those recorded in Table III.

The properties of our material bring to mind the specific bacterial substances studied by Avery and Heidelberger on account of the content in carbohydrates, but it differs by virtue of its antigenic

activity and the presence of protein. Consequently efforts were made to determine whether a specifically reacting non-antigenic substance—a haptene according to our nomenclature—could be separated. In this we succeeded in the following manner.

The first alcoholic extract, richer in protein, was discarded. 1 gm. of dry substance obtained in the second extraction was taken up in 10 cc. of water and

TABLE I.

Precipitation Tests.

Antigens: A = crude extract; B = purified product obtained from A in the manner described.

To 0.2 cc. of the antigen dilutions was added 1 capillary drop of immune serum; readings after 5 minutes and 1 hour. The immune serum was obtained by injections of cholera vibrios into a rabbit.

The agglutinin titer of this serum was 1:8000. The intensity of the reactions is indicated as follows: f. tr. = faint trace; tr. = trace; ±, +, +±, etc.

	Antigen	Antigen diluted 1					Readings after
		5000	50,000	250,000	500,000	1,000,000	
Cholera immune serum No. 82	A	++±	+	tr.	0		5 min.
		++++	+±	±	f. tr.	0	1 hr.
	B	+++	+	tr.	0		5 min.
		++++	++	±	tr.	0	1 hr.
Normal rabbit serum	A	0	0				1 hr.
	B	0	0				1 hr.

In 24 hour readings the precipitate seemed to be partly dissolved especially in the tubes with higher concentrations of antigen.

to it was added 40 cc. of N/10 NaOH. After about 1 hour, the solution was centrifuged from some insoluble material, and 3 volumes of alcohol added. In the supernatant fluid which was kept for several hours, a second precipitate formed on addition of 1 more volume of alcohol. The first precipitate was dissolved in 25 cc. of water, neutralized, and a little Na_2CO_3 added whereupon a slight precipitate formed. After centrifuging the supernatant fluid was acidified (weakly acid to Congo red) with acetic acid so that on addition of 2 volumes of alcohol a flocculating precipitate appeared. The precipitate was washed with 95 per cent alcohol and dried with absolute alcohol and ether. The yield was about 0.4 gm.

TABLE II.

Precipitation Tests

The antigens used are the crude bacterial extracts prepared in the manner described. Immune serum 32 was obtained by 3 injections each of 2 0 mg. of the crude cholera extract into a rabbit.

Extract prepared from	*V. choleræ* immune serum 82					*V. choleræ* extract immune serum 32					*B paratyphosus* B immune serum 74					Normal rabbit serum	
	Antigen diluted 1																
	1000	5000	50,000	250,000	500,000	1000	5000	50,000	250,000	500,000	1000	5000	50,000	250,000	500,000	1000	5000
V. choleræ (Krumwiede)	++++	++++	++	±	f. tr.	++++	++++	+	±	0	0	0	0	0	0	0	0
V. choleræ (Wadsworth)	++++	+++	tr.	f. tr.	0	++++	++±	±	0	0	0	0	0	0	0	0	0
V. metchnikovi	f. tr. ?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. tyrogenus	f. tr. ?	0	0	0	0	f. tr. ?	0	0	0	0	0	0	0	0	0	0	0
V. Finkler-Prior	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyphosus B	0	0	0	0	0	0	0	0	0	0	++	+++	+±	tr.	f. tr.	0	0

This product was a white powder, slowly swelling and dissolving in water, yielding a faintly turbid liquid in a 1 per cent solution. On addition of a trace of alkali it dissolved more readily. The solution

TABLE III, *a*.

Precipitation Tests.

Rabbits injected with A = crude extract: B = purified product obtained from A in the manner described.

Injected with	Rabbit No.	Substance A diluted 1:								Read after
		500	5000	50,000	125,000	250,000	500,000	1,000,000	2,000,000	
A	32	–	++++	+	±	tr.	0			1 hr.
		–	++++	+++	+±	±	tr.	0		24 hrs.
	33	–	+++	+	tr.	0				1 hr.
			++++	+++	+±	±	f. tr.	0		24 hrs.
	41	–	++++	+	±	f. tr.	0			1 hr.
			++++	++	+	±	f. tr.	0		24 hrs.
B	90	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	91	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	92	0	0	0						1 hr.
		f. tr.	0	0						24 hrs.
V. choleræ	82	–	++++	+±	±	±	f. tr.	0		1 hr.
		–	++++	+++	++	+	±	tr.	0	24 hrs.
Normal rabbit serum	1	0	0	0						1 hr.
		0	0	0						24 hrs.
	2	0	0	0						1 hr.
		0	0	0						24 hrs.

gave a negative biuret and Millon's reaction, a very slight xanthoprotein reaction, faint traces of turbidity with trichloracetic, tannic acid, and sulfosalicylic acid, and no turbidity with phosphotungstic acid.

With Molisch's reagent it reacted intensely. A 2.5 per cent solution in N/50 sodium hydroxide gave a rotation of +.16 in a 5 cm. tube. An

TABLE III, *b*.

Agglutination Tests.

To 0.5 cc. of the stated dilutions of inactivated serum was added 0.5 cc. saline suspension of heat-killed *V. choleræ* grown for 18 hours on agar slants. The tubes were kept for 2 hours at 37° and overnight in the ice box. A = crude extract; B = purified product obtained from A in the manner described.

Injected with	Rabbit No	Serum diluted 1:							Read after
		50	500	1000	2000	4000	8000	16,000	
A	32	–	++++	+++	++±	+	0		2 hrs.
			++++	++++	+++	+±	±	0	24 hrs.
	33	–	+++	+++	+	f. tr.	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
	34	–	++++	+++	+±	±	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
B	90	0	0	0					2 hrs.
		0	0	0					24 hrs.
	91	0	0	0					2 hrs.
		0	0	0					24 hrs.
	92	0	0	0					2 hrs.
		0	0	0					24 hrs.
V. choleræ			++++	++++	+++	+±	tr.	0	2 hrs.
			++++	++++	++++	+++	+	0	24 hrs.
Normal rabbit serum	1	0	0	0					2 hrs.
		0	0	0					24 hrs.
	2	0	0	0					2 hrs.
		0	0	0					24 hrs.

analysis gave the following values calculated for ash-free material: C, 49.05; H, 7.17; N, 4.34; P, 1.67; no S; ash, 4.98. The second precipitate mentioned above, after reprecipitation with alcohol in acid

solution, analyzed as follows: C, 47.04; H, 7.09; N, 4.31; P, 1.66: ash, 2.84.

When hydrolyzed with N/2 HCl, sugar could be demonstrated by Fehling's solution and by the osazone test, and a precipitate appeared as in the experiments mentioned above.

With cholera immune serum it was precipitated to the same titer as the original product but rather more intensely (Table I). The precipitin reaction was not diminished by heating a 2 per cent solution for 1 hour in the steam bath; neither was it appreciably affected by digestion with pepsin or trypsin. It resisted the action of nitrous acid.

The specificity of the substance was investigated with the results shown in Table II.

The tests demonstrate the specificity of the reaction with the precipitable substance. A third strain of cholera gave a product which reacted only weakly with cholera immune serum. This strain was atypical in that it showed spontaneous agglutination and was considerably less agglutinated by immune serum than the other strains.

The antigenic activity of the crude and purified extract was tested as follows:

Two batches of rabbits were injected—one with the crude material used for the purification; another with the purified substance. Three injections each of 2 mg. of the substances dissolved in 1 cc. saline were given intravenously at intervals of 5 days. 7 days after the last injection the sera were tested. The results are presented in Tables III, *a* and III, *b*. Before the injections the sera of the animals did not agglutinate in dilutions of 1:10 and 1:50 and gave no precipitin reactions.

It appears from the experiment that substance B while reacting strongly *in vitro* (Table I) had little if any antigenic activity.

SUMMARY.

The investigations described show that it is possible to extract specific substances from *V. choleræ* by means of hot dilute alcohol. This result cannot be attributed to the presence of bacilli in the extracts as would follow from the statements made by Prausnitz in his criticism of the work of Levaditi.

The original extract contains protein and exhibits antigenic properties. From this preparation an almost protein-free product was obtained. It was fully active in the precipitin test but had lost almost completely the antigenic activity. Accordingly the latter preparation belongs to the class of substances described by Zinsser as residue antigens and studied chemically by Avery and Heidelberger. The fact that the immune sera resulting from the injections of the crude extracts acted upon the non-immunizing precipitable substance indicates that in the crude extract there is present an antigenic complex consisting of protein and the specific substance.

Regarding its chemical nature it follows from the foregoing that the precipitable but non-immunizing substance is not a protein. On hydrolysis it yielded a considerable quantity of sugar although less than that given for the specific carbohydrates of pneumococci and *B. friedlænderi*. The product contains nitrogen and phosphorus and on hydrolysis a substance of acid character separates from the solution. Accordingly the substance prepared would appear to have either a rather intricate structure or to be a complex carbohydrate, similar to those described by Avery and Heidelberger, but still containing impurities. This issue can probably be decided by further studies.

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[Reprinted from The Journal of Immunology, July, 1927, Vol. xiv, No. 1, pp. 81–83.]

ON THE HETEROGENETIC HAPTENE.

Fourth Communication.[1,2,3]

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(Received for publication, June 28, 1927.)

In attempting to purify the specific part of the heterogenetic haptene, present in alcoholic extracts of horse kidneys, fractions were obtained which are distinguished from the common lipoids by their solubility in water. The material of one fraction described recently[3] is practically insoluble in alcohol, chloroform and ether and gives a viscous solution in hot water. With orcinol and copper it gives no purple color reaction in contrast to other water-soluble fractions; it contains neither phosphorous nor sulfur.

It was possible to obtain from this serologically active product two fractions differing in their composition. The method of preparation was the following. The crude material separating from alcoholic extracts of horse kidneys and freed from ether soluble substances[1] was dissolved in 20 parts of hot pyridine and allowed to stand in the ice box for forty-eight hours. After removing the sediment the filtrate was evaporated under reduced pressure to nearly dryness. The residue was dissolved in chloroform and poured into acetone. The precipitate was filtered off and dried. The dry material was extracted with 10 parts of a mixture of equal parts of chloroform and of methyl alcohol. This procedure was repeated. The residue was taken up in 100 parts of hot water and placed in a shaking machine for several hours; a small fraction remained undissolved.

To the solution half the volume of Fehling's solution was added. A gelatinous precipitate was formed which was washed with a small

[1] Jour. Immunol., 10, 731, 1925.

[2] Proc. Soc. Exper. Biol. and Med., 23, 343, 1926.

[3] Proc. Soc. Exper. Biol. and Med., 24, 693, 1927.

volume of water. It was then suspended in water and acidulated with hydrochloric acid. To the opalescent solution alcohol and ether, or acetone was added to precipitate the material. The product obtained in this manner was a white powder having the following composition: C = 55.39 per cent, H = 9.22 per cent, N = 2.15 per cent.

The residue which remained after extraction with hot water, as mentioned above, was again taken up in water and boiled until nearly all dissolved. The mixture was centrifugalized, to the clear solution Fehling's solution was added, and the copper salt dissolved in a little 10 per cent hydrochloric acid. To the solution just enough acetone was added to bring about precipitation. This material was a white powder. Dried to constant weight at 100° under reduced pressure it analyzed as follows: C = 40.91 per cent, H = 6.78 per cent, N = 1.79 per cent.

Thus the elementary composition of the products obtained differs considerably from that of the known lipoids. Both substances were serologically active. The substance with lower C content gave complement fixation with heterogenetic immune serum up to 1:20,000,000, using a method similar to that described previously (addition of 5 parts of crude sphyngomyelin). Immunization experiments were made with this product by injecting into 5 rabbits 5 cc. of a saline solution containing 1 mgm. of the substance and 0.5 cc. pig serum at intervals of four or five days. The tests with the sera seven days after the third injection showed the formation of hemolysins for sheep blood. Before the immunization none of the sera hemolyzed completely in the dilution 1:25. After three injections the five sera hemolyzed completely in the dilutions: 1:100, 1:200, 1:400, 1:400, 1:400, respectively (0.5 cc. diluted serum, 0.5 cc. $\frac{1}{10}$ guinea-pig serum, 1 drop 50 per cent sheep blood, one hour at 37°).

The substance with low C content was hydrolyzed by heating 100 mgm. for twenty-four hours with 5 cc. of 3 per cent sulfuric acid at 100°. The product of hydrolysis contained some water-insoluble material. The aqueous solution titrated with Fehling's solution gave a reduction corresponding to 28 mgm. glucose. It contained 2.2 mgm. N (microanalysis). The insoluble material was taken up

in acetone leaving a small insoluble black residue. The acetone solution on concentration left a residue which on microcombustion gave the following values: C = 71.49 per cent, H = 11.81 per cent.

The composition of our preparations suggests the possibility that they contain specific groups similar to those of the bacterial haptenes studied by Avery and Heidelberger.

[Reprinted from The Anatomical Record, May 25, 1927, Vol. xxxv, No. 3, pp. 149–159.]

A QUANTITATIVE STUDY OF THE GOLGI APPARATUS IN SPINAL GANGLION CELLS.

By W. P. COVELL.

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The first paper of this series (Covell, '26) dealt with the nucleo-cytoplasmic ratio in the renal tubule. It was found that the ratio was characteristic for each segment and that it was greater in the convoluted tubules than in the limbs of the loop of Henle.

Some observations with Doctor Cowdry, which will soon be reported, show that this change is accompanied by a distinct reduction in the mitochondria-cytoplasmic ratio. It is well known that the cells of the different segments are likewise physiologically different in the part which they play in urinary secretion, in their response to poisons, vital dyes, and other experimental methods of analysis. What these modifications in cellular structure mean we do not know, but it seems profitable to attempt to ascertain which elements, or ratios, vary dependently and which vary independently; in other words, to study the integrative action of the cell.

Obviously, the relations may be expressed in terms of surface or of volume, so that one has some ten or more variables to deal with. It is interesting to note, in this connection, that Ponder ('26) has established the existence of an interesting and perhaps significant relation between the surface area of the surfaces of the nucleus, zymogen granules, and mitochondria count. du Noüy and Cowdry ('27) have attempted to study the surface of the nucleus, zymogen granules, and mitochondria in the acinous cells of the pancreas.

The Golgi apparatus, however, seems to have been wholly neglected. It is with it that this paper is concerned. At first, attempts were made to study this structure in the cells of the renal tubule and to correlate the findings with the observations referred to, but this proved impracticable for several reasons. The strands of the network in these cells are of variable thickness, making quantitative estimation difficult and there was always the question whether all of the material actually

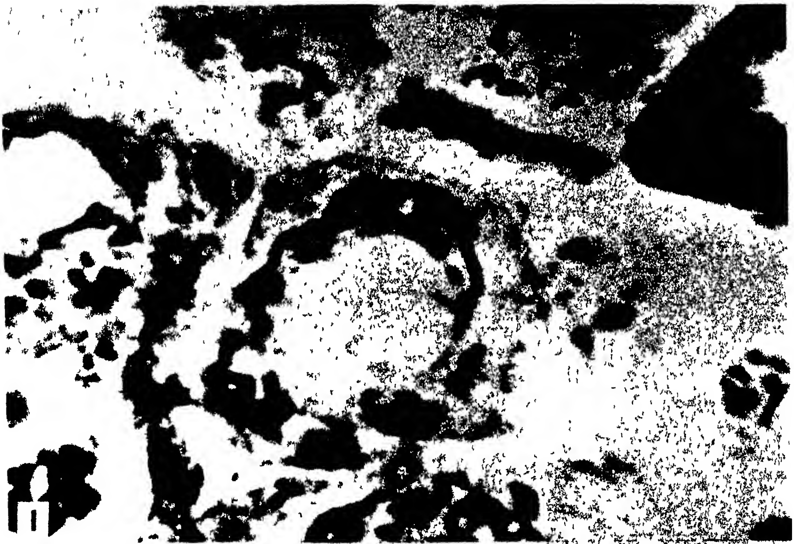
present was faithfully preserved. In order, at least, to work under the most favorable conditions, the spinal ganglion cells of young mammals were chosen as material, because in them the Golgi apparatus may be demonstrated with unparalleled clearness and constancy.

MATERIAL AND METHODS.

Ganglia in the cervical and lumbar enlargements were removed from seven rabbits two and a half to four weeks of age and from one adult rabbit, and fixed for twenty-four hours in Kolatchev's fluid, as modified by Nassonov ('24). They were then transferred to 2 per cent osmic acid and kept at 37½°C. for eight to nine days. Following dehydration and clearing, the tissues were embedded in paraffin and sections were cut 5 μ in thickness.

Every effort was made to employ photographic methods, and good photomicrographs were secured, which illustrate the general appearance of Golgi apparatus (figs. 1 and 2); but the photographs could not be used as a basis for the detailed measurements required, because they give information only concerning a single optical plane, whereas the whole depth of the section must be taken into consideration. The photographs are, furthermore, imperfect in the sense that the outlines of the Golgi apparatus in them are less distinct than in the original preparation, for the reason that strands of the apparatus which lie above and below the optical plane of the photomicrograph are blurred. An Edinger projection apparatus was tried, but did not prove helpful, because constant checking up of the projected outlines with the actual preparation was impossible.

Accordingly, the more laborious method of making drawings at the table level with a camera lucida was adopted, with an optical combination yielding a magnification of 3050 diameters. In this way twenty-five cells of various sizes were examined, the boundaries of the cell, of the nucleus, and the Golgi apparatus being outlined. Every section of a cell was drawn, and since the number of sections was dependent upon the size of the cell, this proved a useful means of checking up the calculated diameters. The mean diameter of the cell was obtained by measuring in microns several arbitrarily chosen dimensions of the drawing of the largest section of the cell and by dividing the figure thus obtained by the magnification. The cell volume and sur-



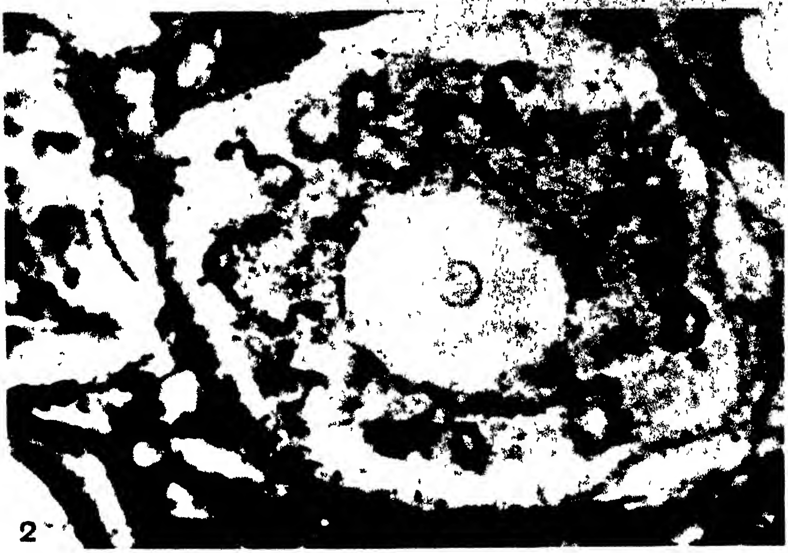


Fig. 1. Photomicrograph of Golgi apparatus in small-sized spinal ganglion cell × 2000

Fig. 2. Photomicrograph of Golgi apparatus in large-sized spinal ganglion cell. × 2000.

face were then computed on the assumption that the cell was spherical in form. The nucleus of each cell was treated in a similar manner. An error, however, unavoidably remains through the impossibility of measuring the cell processes, but it is probably not a large one.

The surface and volume of the Golgi apparatus were computed as for a tube of constant diameter. As a result of many measurements, this diameter was found to be about 0.4 μ. The length was quickly and accurately measured by employing a map-measuring machine, manufactured by Keuffel & Esser. This instrument is commonly used by cartographers. It consists essentially of a small wheel which is run over the outlines and which registers the distance traveled on a dial, in both centimeters and inches. Obviously, the actual length is somewhat greater than the length as measured in this way, because the strands extend at various angles through a depth equal to the thickness of the section, so that they have a certain vertical length added to the length projected on the single plane of the drawing. It is recognized also that the measurements apply only to cells prepared by this method, not to living cells. The volume of fluid displaced by the nervous tissue after fixation, dehydration, clearing, etc., was found to be 14 to 18 per cent greater than the amount displaced by the same tissue before fixation. Because the various cellular components respond differently to fixatives, a certain differential shrinkage (or swelling) may conceivably occur; but this must be set aside for the present.

RESULTS.

The data thus secured were treated in several different ways. When the surface area and the volume of the Golgi apparatus were plotted against the diameter of the cell, a tendency toward a shallow concave curve resulted in each instance (figs. 3 and 4). This curve was found to be approximated by means of the formula, $Y = a X^b$, where Y is the surface area or the volume of the Golgi apparatus, X the diameter of the cell in μ, and a and b constants, empirically determined. The data were fitted to curves by means of the method of averages. A more perfect correspondence was obtained by eliminating cell no. 21 (table 1) from the calculations, although the observations for this cell are included in figures 3 and 4.

Various ratios were computed in order to ascertain if the surface or volume of the Golgi apparatus varied relatively with the surface or volume of the cell or nucleus and the volume of the cytoplasm. Thus the computed surface area of the Golgi apparatus was divided

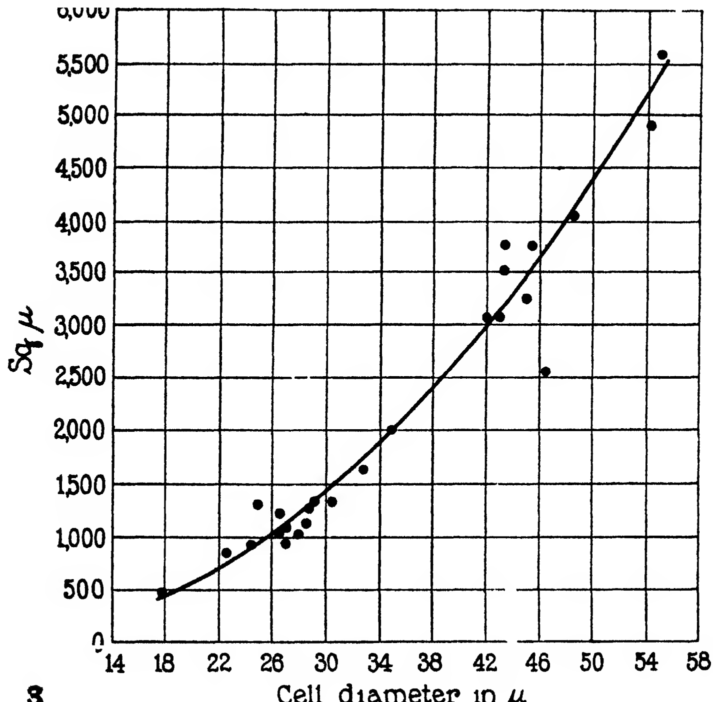


Fig. 3. Graph with curve illustrating the relationship of surface area of Golgi apparatus to cell diameter. Abscissae: cell diameter in microns. Ordinates: surface area of the Golgi apparatus in sq μ. Individual observations indicated by dots.

by the computed surface area of the cell and likewise by that of the nucleus.

In table 1 the cells are arranged according to their size. They range in diameter from 17.86 μ to 54.92 μ. The nuclear diameter varies from 9.67 μ to 19.67 μ. The surface of the Golgi apparatus

varies from 476.18 sq.μ in the smallest cell to 5587.72 sq.μ in the largest cell and the volume from 58.55 cu.μ to 691.97 cu.μ.

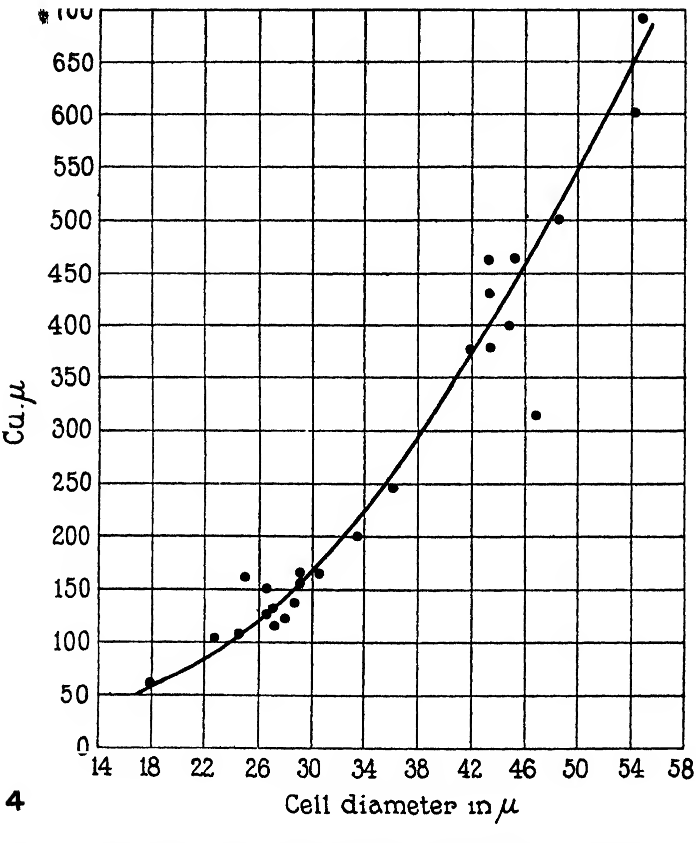


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Fig. 4 Graph with curve illustrating the relationship of volume of Golgi apparatus to cell diameter. Abscissae cell diameter in microns. Ordinates: volume of Golgi apparatus in cu.μ. Individual observations indicated by dots.

The surface area of the Golgi apparatus divided by the surface area of the cell gives a figure of fair agreement for all twenty-five cells on which determinations were made. The mean ratio is 0.511, or the surface of the Golgi apparatus is roughly one-half as great as

the surface area of the cell. The absolute departure of all of the ratios from the mean is 0.053 and the relàtive deviation is 10.5 per cent.

TABLE I.

NO OF CELL	CELL DIAMETER	NUCLEAR DIAMETER	GOLGI APPARATUS		SURFACE OF GOLGI APPARATUS / SURFACE OF CELL	SURFACE OF GOLGI APPARATUS / SURFACE OF NUCLEUS	SURFACE OF GOLGI APPARATUS / VOLUME OF CYTOPLASM × 100	VOLUME OF GOLGI APPARATUS / VOLUME OF CELL × 100	VOLUME OF GOLGI APPARATUS / VOLUME OF CYTOPLASM × 100
			Surface area	Volume					
	μ	μ	sq μ	cu μ					
1	17 86	9 67	476 18	58 55	0 475	1 62	18 96	1 96	2 33
2	22 78	11 31	849 02	104 39	0 521	2 11	15 62	1 68	1 92
3	24 59	12 13	910 82	111 98	0 479	1 97	13 27	1 44	1 63
4	25 08	11 96	1310 50	161 13	0 663	2 92	17 79	1 95	2 19
5	26 55	10 65	1043 54	128 30	0 471	2 93	11 39	1 31	1 40
6	26 64	12 62	1220 84	150 10	0 547	2 44	13 80	1 52	1 69
7	27 04	13 28	1093 18	134 41	0 476	1 97	11 98	1 30	1 47
8[1]	27 21	10 33	947 29	116 47	0 408	2 83	9 51	1 10	1 17
9	28 03	13 11	1013 15	124 56	0 411	1 88	9 79	1 08	1 20
10	28 52	13 11	1134 22	139 45	0 444	2 10	10 34	1 15	1 27
11	28 80	13 11	1288 72	158 45	0 494	2 39	11 37	1 27	1 40
12	29 18	14 10	1339 88	164 74	0 501	2.14	11 61	1 26	1 43
13	30 40	12 62	1345 96	165 48	0 463	2 59	9 85	1 13	1 21
14	33 67	14 10	1633 70	200 86	0 459	2 61	8 83	1 00	1 09
15[1]	35 08	15 73	2016 16	247 89	0 521	2 59	9 80	1 10	1 20
16	41 96	15 00	3081 48	378 87	0 557	4 36	8 35	0 98	1 03
17	43 44	15 08	3097 69	380 86	0 522	4 30	7 53	0 88	0 92
18	43 60	15 08	3768 91	463 39	0 631	5 27	9 06	1 07	1 11
19	43 60	17 04	3520 68	432 87	0 589	3 86	8 63	0 99	1 06
20	45 08	14 75	3262 33	401 11	0.511	4 78	7 05	0 84	0 87
21	45 49	16 39	3789 17	465 88	0 583	4 50	8 07	0 94	0 99
22	46 55	16 06	2571 87	315 59	0 378	3 17	5 08	0 60	0 62
23[1]	48 52	17 38	4067 78	500 14	0 550	4 28	7 13	0 83	0 87
24	54 16	15 90	4908 69	603 53	0 532	6 18	6 05	0 72	0 74
25[1]	54 92	19 67	5587 72	691 97	0 589	6 75	4 60	0 79	0 83

[1] Cell of an adult rabbit of 2450 grams body weight.

The surface area of the Golgi apparatus divided by the surface area of the nucleus gives a ratio which varies with the size of the cell.

Thus, in the smaller ganglion cells the surface of the Golgi apparatus is from one and one-half to three times that of the nucleus, and in the largest cells it is even greater in proportion, ranging from about four to nearly seven times.

The amount of cytoplasm, in terms of volume, when divided into the figure for the surface area of the Golgi apparatus and multiplied by 100 gives a ratio which decreases in magnitude as the cell size increases. There is, then, relatively greater surface area of Golgi apparatus per volume of cytoplasm in the smaller cells than in the larger.

The volume of the Golgi apparatus divided by the volume of the cell and likewise by the cytoplasmic volume and multiplied by 100 gives ratios which decrease in magnitude with an increase in cell or cytoplasmic volume. The ratio pertaining to cell volume is less than that for cytoplasmic volume, since the volume of the nucleus is not considered in the latter ratio.

Figure 3 is a shallow concave curve resulting from the plotting of the surface area of the Golgi apparatus against the diameter of the cell. This curve may be approximately expressed by means of the formula:

$$\text{Surface area of the Golgi apparatus in sq } \mu \ (\times 100) = 68.72 \ (\text{cell diameter, } \mu)^{2\ 234}$$

The absolute deviation of the observed from the calculated values is 160.5 sq.μ and the relative deviation is 8.2 per cent. The calculated surface area of the Golgi apparatus for a cell of 20 μ in diameter is 553.9 sq.μ. For one of 35 μ it is 1933.2 sq.μ and in a cell of 50 μ the surface area is 4291.0 sq.μ.

In figure 4 the volume of the Golgi apparatus has been plotted against cell diameter. This curve may be numerically expressed in approximate terms by means of the empirical formula:

$$\text{Volume of the Golgi apparatus in cu.} \mu \ (\times 100) = 8.738 \ (\text{cell diameter, } \mu)^{2\ 234}$$

The calculated volume of the Golgi apparatus of a cell of 20 μ in diameter is 70.4 cu.μ, for one of 35 μ it is 245.9 cu.μ, and in a cell of 50 μ in diameter it is 545.8 cu.μ. The average absolute deviation of the observed from the calculated values is 19.7 cu.μ. The percentage deviation is 8.9.

DISCUSSION.

The measurements seem to indicate that the Golgi apparatus is susceptible of quantitative study. The surface which the apparatus affords, in spinal ganglion cells, is about one-half as large as the total surface of the cell. The relaton of surface area of Golgi apparatus to cell diameter may, indeed, be expressed in the form of a curve which is numerically approximated by means of the formula for a simple parabola, $Y = a X^b$ (fig. 3). What this may signify I am at present wholly unable to say. Such a definite relation does not apparently exist between the Golgi apparatus and the nucleus (table 1).

These observations have, of necessity, been made on cells impregnated with osmium. The condition of the Golgi apparatus in the living cells of mammals is unknown. Parat ('24) and his associates state that it occurs in the form of a series of vacuoles capable of being stained with neutral red. The principal evidence advanced consists of a topographic correspondence between the vacuoles in supravitally stained cells and the blackened networks brought to light in fixed cells by the action of osmic acid. The actual changes in the vacuoles resulting from fixation and leading to the formation of a typical Golgi apparatus have never been followed. Quantitatively, a difference seems to exist, because in nerve cells the neutral-red vacuoles are not particularly abundant, while the Golgi apparatus, as revealed in osmium preparations, is unusually highly developed. Further information is urgently needed. Whether or not it is represented by a series of vacuoles, the general configuration of the Golgi apparatus in the living cell must correspond substantially with that revealed by methods of impregnation with osmium, because by counterstaining these preparations it may be observed that the mitochondria (which are easily observed during life) are not noticeably displaced. There have been no massive movements of cellular contents.

As to the function of the Golgi apparatus, very little may be said. It seems clear, however, that there is some measure of discontinuity between its substance and that of the surrounding cytoplasm; in other words, that a gradual transition from one to the other does not occur. Such a surface or interface between materials of different character may be active in processes of adsorption and synthesis—as the mito-

chondria-cytoplasmic surface film is believed to be (Cowdry, '26)—though in a different way, depending upon the physicochemical composition of the surface.

Very recently, it has been stated that the Golgi apparatus is definitely concerned in the formation of secretion antecedents in gland cells (Nassonov, '24; Bowen, '26, etc.). A few years ago this rôle was just as forcibly attributed to the mitochondria, and at a still earlier date to the nucleus and certain extranuclear substances, presumably derived from it. All of which is probably true up to a certain point and nothing less than one would expect, for, in discharging its principal function, it is natural to suppose that the cell mobilizes its entire machinery. But it is too early to disconnect in our imagination, with any measure of confidence, this machinery and to assign definite and exclusive rôles to the Golgi apparatus, mitochondria, or any other cellular components.

SUMMARY AND CONCLUSIONS.

1. The Golgi apparatus may be studied quantitatively in successful preparations of spinal ganglion cells.
2. The computed surface area of the Golgi apparatus is approximately one-half as large as the total cell surface.
3. There is relatively a greater surface area of Golgi apparatus in small cells in relation to cytoplasmic volume than in large cells.
4. In small cells there is relatively less Golgi apparatus surface in relation to nuclear surface than in large cells.
5. There is relatively more volume to the Golgi apparatus of small cells in relation to cell and cytoplasmic volumes than in large cells.

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[Reprinted from The Journal of Experimental Medicine, July 1, 1927, Vol. xlvi, No. 1, pp. 65–80.]

EFFECT OF HOST IMMUNITY TO A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE RABBIT NEOPLASM.

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(Received for publication, March 22, 1927.)

In a previous paper (1) experiments were reported in which it was shown that 2 filterable viruses, Virus III and vaccine virus, grow and survive for unusually long periods in a transplantable malignant neoplasm of the rabbit. Early in the course of this work, it was found that the tumor (2) was infected with Virus III (3) and was being transferred with it, and also that the rabbits so inoculated became immune to Virus III. Since Virus III is indigenous to rabbits and its incidence fairly high judging from the number of immune animals in the normal stocks of these laboratories (15 to 20 per cent on an average), it is impossible to say when the tumor became infected. In any event, the question arose as to whether growth and malignancy of the tumor were affected first, by the presence of the virus, and second, by host immunity to the virus. An opportunity to investigate these problems was afforded by the fact that we were able to obtain the tumor without the virus and to transplant it in the same manner as the stock tumor bearing the virus.

Experiments are reported in the present paper dealing with the effect of the immunity of the host to Virus III on the pathological processes induced by both the virus-bearing and the virus-free tumor strains. A subsequent paper deals with the question of the effect of the virus on the growth and malignancy of the tumor (4).

EXPERIMENTAL.

Materials and Method.

Neoplasm.—The tumor employed in these experiments has been studied ex tensively ever since its recognition in October, 1920, and its subsequent trans-

plantation, and a number of papers dealing with various aspects of the subject have been published (2). It suffices to state here that the tumor is considered to be of epithelial origin composed of cells allied to those found in the bulb and root sheath of the hair, and that it has been transplanted through successive generations by intratesticular inoculations. This method of inoculation has proven effective, not only from the standpoint of obtaining actively progressing primary tumors, but also for the study of the pathological process as a whole. The manifestations of the disease vary markedly both in individual rabbits of a series and between groups of animals inoculated at one time as compared with those inoculated at another. The growth and fate of the primary tumor, the incidence of metastases, the distribution, number, extent and state of the growths and the mortality rates, both actual and estimated, are among the variable features of the disease which must be taken into account in evaluating the character of the process at any time.

It was found in October, 1924, that all rabbits inoculated with the tumor became immune to Virus III, and by means of rapid passage of emulsions of primary tumors or metastases, the presence of the virus was regularly demonstrated.

Virus III—While attempting to produce chicken-pox in rabbits, a filterable transmissible agent was discovered (3) This agent produces gross as well as microscopic lesions in the cornea, skin and testicles of rabbits, and an infection with it leads to an immunity against subsequent infections with the same material. For convenience, this agent has been spoken of as Virus III. At first it was considered not unlikely that the virus is the etiological agent of varicella. Further work, however, disclosed the fact that Virus III is indigenous to rabbits and that it is as typical a virus as vaccine virus or the virus of herpes simplex from both of which it can easily be differentiated.

Virus-Free Strain of Tumor—This strain was obtained from a rabbit inoculated with the stock tumor bearing the virus. The animal died several weeks after inoculation and accidentally lay in a warm room 12 to 18 hours. The primary tumor was removed and inoculated into the testicles of 3 rabbits. Although the development of these transplants was much delayed, growth later occurred and transfers were successfully made Many generations of this strain of the tumor have been studied, and it has been impossible to demonstrate the presence of Virus III by methods which suffice with the stock tumor. Rabbits inoculated with this strain, moreover, do not become refractory to skin infection with Virus III, their sera do not become virucidal and no nuclear inclusions, typical of Virus III reactions, have been found in young tumors. The virus-free state of this strain was controlled from time to time by appropriate tests.

Conduct of Experiments.—The experiments were carried out from February, 1925, to January, 1926. Groups of 10 male rabbits from selected stocks were immunized to Virus III by a single intracutaneous or subcutaneous injection of testicular emulsions containing the virus. At intervals of 20 to 39 days after the injection of Virus III, the rabbits were inoculated in one testicle with 0.3 cc. of a

salt solution emulsion of an actively growing primary tumor. Comparable groups of non-immunized rabbits were inoculated at the same time. Both the stock tumor strain bearing the virus and the virus-free strain of tumor were used. The total number of rabbits employed in the experiments reported in this paper was 129, 49 of which were immunized.

The rabbits were separately caged and fed the same diet of hay, oats and cabbage. Frequent examinations were made, special attention being paid to the general physical condition of the animals, the character and course of the primary tumor and the development of secondary growths in superficial parts of the body.

The experiments were terminated 2 months after inoculation at which time all surviving animals were killed by an injection of air in the marginal ear vein. This period was selected upon the basis of previous experience as being sufficient to include a large proportion of the deaths due to tumor growth, and at the same time, sufficient to allow for the recovery of many rabbits. Rabbits which developed a pronounced cachexia or paralyses during the observation period were killed at that time Each animal was subjected to postmortem examination, particular attention being given to the state of the primary tumor and to the distribution, number and condition of secondary growths together with an estimation of the degree of organ involvement.

Method of Analysis of Results.—The data obtained from clinical observations and postmortem examinations have been analyzed upon a group basis. The actual deaths from the tumor process have been classified in 2 groups upon the basis of postmortem findings. In one, designated as "malignant," the widespread or significant distribution of tumor was such that there could be no question that the malignant process was responsible for the death of the animal. In the other, designated as "accidental," the distribution of tumor was usually more limited, and except that a site such as the spine or jaws was involved, it has been assumed that death would not have occurred at this time. It is obvious that in an estimation of degrees of malignancy based upon comparative mortality rates, the numbers of accidental fatalities possess far less significance than those in the category of malignant deaths.

A considerable number of rabbits survived the observation period of 2 months. In some of them, however, the distribution of metastases was such that it is probable that death would eventually have occurred as a result of the tumor process Growths in both suprarenal glands or in the facial and jaw bones are instances of this type of disease. These cases have been classified as "probable deaths" On the other hand, there were instances of surviving animals in which a few foci of tumor were found, but upon the basis of the distribution and state of these growths, they have been classified as "probable recoveries."

The number of foci of metastases refers to the number of organs or tissues involved. not to the actual numbers of secondary growths, and consequently, the expressions "foci of metastases," "distribution of metastases" or "metastatic rate" are used rather than "number of metastases"

TABLE I.

Virus-Free Tumor in Normal and Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality: Total		Mortality: Malignant cases		Mortality: Accidental deaths		Metastases: Incidence		Metastases: Total foci	Metastases: Relative rate	Metastases: Actual rate	No. of metastatic foci: Deaths	No. of metastatic foci: Survivors		Probable deaths	Actual and probable recoveries	
					No.	Per cent	No.	Per cent	No	Per cent	No	Per cent								No.	Per cent
		days																			
I	N		9	All +	3	33 3	2	22 2	1	11.1	6	66.7	62	6 9	10 3	14, 20, 22	3, 2,	0 in 5	1	5	55.5
	I	39	9	All +	1	11.1	1	11.1	0	—	3	33 3	23	2 6	7.7	19	2, 2	0 in 6	1	7	77.7
II	N		10	All +	1	10 0	1	10 0	0	—	6	60 0	41	4 1	6 8	25	4, 4, 3, 3, 2	0 in 4	1	8	80.0
	I	25	9	8 +	1	11 1	0	—	1	11 1	5	55 5	21	2 3	4 2	11	7, 1, 1, 1	0 in 4	1	7	77 7
III	N		10	All +	1	10 0	1	10 0	0	—	4	40 0	32	3 2	8 0	21	8, 2, 1	0 in 6	1	8	80.0
	I	28	11	All +	0	—	0	—	0	—	6	55 5	20	1 8	3 3	—	7, 6, 3, 2, 1, 1	0 in 5	0	10	100.0
Total	N		29		5	17 2	4	13 8	1	3.4	16	55.2	135	4 7	8 4	102	32	0 in 15	4	20	68.9
	I		29		2	6.9	1	3 4	1	3.4	14	48.3	64	2 2	4 6	30	34	0 in 15	3	24	82.7

N = normal rabbits; I = rabbits immune to Virus III.

TABLE II.

Virus-Bearing Tumor in Normal and Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality: Total No.	Mortality: Total Per cent	Mortality: Malignant cases No	Mortality: Malignant cases Per cent	Mortality: Accidental deaths No	Mortality: Accidental deaths Per cent	Metastases: Incidence No.	Metastases: Incidence Per cent	Metastases: Total foci	Metastases: Relative rate	Metastases: Actual rate	No of metastatic foci: Deaths	No of metastatic foci: Survivors	Probable deaths	Actual and probable recoveries No.	Actual and probable recoveries Per cent
		days																		
IV	N		10	All +	4	40 0	4	40 0	0	—	6	60 0	101	10 1	16 8	30, 32, 16, 12	9, 2 0 in 4	1	5	50.0
	I	28	10	All +	2	20 0	1	10 0	1	10 0	6	60 0	40	4 0	6 7	25, 6	4, 2, 2, 1 0 in 4	0	8	80.0

N = normal rabbits; I = rabbits immune to Virus III.

TABLE III.

Virus-Bearing Tumor in Normal Rabbits. Virus-Free Tumor in Immune Rabbits.

Experiment	Group	Time after Virus III injection	No of rabbits	Primary tumors	Mortality: Total No	Mortality: Total Per cent	Mortality: Malignant cases No	Mortality: Malignant cases Per cent	Mortality: Accidental deaths No	Mortality: Accidental deaths Per cent	Metastases: Incidence No	Metastases: Incidence Per cent	Metastases: Total foci	Metastases: Relative rate	Metastases: Actual rate	No of metastatic foci: Deaths	No of metastatic foci: Survivors	Probable deaths	Actual and probable recoveries No	Actual and probable recoveries Per cent
		days																		
V	N		10	All +	5	50 0	4	40 0	1	10 0	7	70 0	74	7 4	10 6	21, 17, 16, 9, 7	3, 1 0 in 3	0	5	50.0
	I	39	9	All +	1	11 1	1	11 1	0	—	3	33 3	23	2 6	2 6	19	2, 2 0 in 6	1	7	77 7
VI	N		10	All +	5	50 0	3	30 0	2	20 0	7	70 0	75	7 5	10 7	21, 15, 14, 14, 6	3, 2 0 in 3	0	5	50 0
	I	20	10	9 +	1	10 0	0	—	1	10 0	1	10 0	10*	1 0	10 0	10	0 in 9	0	9	90 0
VII	N		11	All +	4	36 4	3	27 3	1	9 1	9	81 9	74	6 7	8 2	26, 19, 14, 6†	4, 2, 1, 1, 1 0 in 2	0	7	63.6
	I	25	9	8 +	1	11 1	0	—	1	11 1	5	55 5	21	2 3	4 2	7	11, 1, 1, 1 0 in 4	1	7	77 7
VIII	N		10	All +	4	40 0	4	40.0	0	—	6	60 0	101	10 1	16 8	32, 30, 16, 12	9, 2 0 in 4	1	5	50.0
	I	28	11	All +	0	—	0	—	0	—	6	55 5	20	1 8	3 3	—	7, 6, 3, 2, 1, 1 0 in 5	0	11	100.0
Total	N		41		18	43 9	14	34 1	4	9 8	29	70 4	324	7 9	11 0	70	29 0 in 12	1	22	53 7
	I		39		3	7 7	1	2 6	2	5 1	15	38 5	74	1 9	4 9	36	37 0 in 24	3	33	84 6

N = normal rabbits; I = rabbits immune to Virus III.

*Number of metastatic foci estimated. † Complicating empyema.

TABLE IV.

Virus-Free Tumor in Normal Rabbits. Virus-Bearing Tumor in Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumor	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
					Total		Malig-nant cases		Acciden-tal deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors			
					No	Per cent	No	Per cent	No	Per cent	No	Per cent							No	Per cent
		days																		
IX	N		10	All +	1	10 0	1	10 0	0	—	4	40 0	32	3 2	6 7	21	8, 2, 1 0 in 6	0	9	90.0
	I	28	10	All +	2	20 0	1	10 0	1	10 0	6	60 0	40	4 0	8 0	25, 6	4, 2, 2, 1 0 in 4	0	8	80 0

N = normal rabbits, I = rabbits immune to Virus III.

The distribution of secondary growths has been considered upon both a relative and an actual basis, the former including all animals of a group, while the latter takes into account only those rabbits in which metastases were found. The actual rate is obviously accentuated by individual rabbits with large numbers of foci, so that from a group standpoint the relative rate is a fairer index of comparative metastatic involvement. For other purposes, such as a numerical comparison of the uniformity of tumor distribution, both rates are of value.

Results.

The results of 9 experiments consisting of both clinical and postmortem observations are summarized in Tables I, II, III and IV.

Table I contains data obtained in 3 experiments in which the virus-free tumor was inoculated in normal rabbits and in rabbits injected 25, 28 or 39 days previously with Virus III. Table II consists of the data of one experiment in which the virus-bearing tumor was used in an immunized group of rabbits; Virus III had been injected 28 days before the tumor. In the next 4 experiments, Table III, the pathological picture in normal rabbits induced by the virus-bearing tumor is contrasted with that of the virus-free tumor in rabbits injected 20, 25, 28 or 39 days previously with Virus III. Finally, Table IV summarizes the results of a single experiment in which the behavior of the virus-free tumor in normal rabbits was compared with that of the virus-bearing tumor in rabbits injected 28 days previously with Virus III.

DISCUSSION.

Before discussing the results of the experiments in which the course and character of the malignant disease in rabbits immune to Virus III was investigated, certain features of this study which must be considered in interpreting and evaluating the results should be briefly mentioned.

Because of the variability in the manifestation of the disease induced by this tumor, and in particular, the seasonal character of these variations (5), it is important in an investigation of comparisons of the disease under diverse conditions to carry out experiments at different seasons of the year. This has been done in the present instance as shown by the following dates of tumor inoculation:

Experiments I and V....................	March 13 and 18, 1925.
Experiment VI..........................	May 12 and 15, 1925.
Experiments II and VII.................	October 27 and 28, 1925.
Experiments III, IV, VIII and IX	November 24 and 25, 1925.

The state of different materials used for inoculation must be considered in comparing results of 2 series, one of which was inoculated with the tumor bearing the virus and the other with the virus-free tumor (Tables III and IV). Both strains were transferred at monthly intervals to groups of not less than 10 rabbits and although actively growing primary tumors were used for this purpose, there was no criterion which would enable one to say that the 2 tumors were alike in actual or potential qualities of growth. But the chance of using less favorable material was the same in one case as in the other, except for the fact, which will be discussed later, that the disease in rabbits immune to Virus III was less malignant than in normal animals, and in certain experiments the virus-free tumor used for inoculation was derived from the primary growth of this strain in an immune rabbit. The possibility that this condition of host immunity modified the growth capacity and malignant potentialities of the tumor cannot be disregarded, but on the other hand, it should be pointed out that there is no reason for assuming that such modifications were of the nature of fixed characteristics.

One must also consider the interval between the inoculation of the normal and of the immune groups of an experiment. This factor does not enter into the first 4 experiments in which both groups were inoculated on the same day or in the last 3 in which inoculations were carried out on succeeding days. But in the 5th experiment there were 5 days, and in the 6th experiment, there were 3 days intervening between the inoculation of the 2 groups. What effect, if any, such a time difference might have upon the course and character of the malignant disease cannot be predicted, and one might be disposed to ignore it because of its shortness as compared with the 2 months' duration of the experiments and attribute any marked divergence of the pathological picture in the 2 groups to other factors. We have repeatedly observed, however, definite variations in the plane or level of malignancy in series of rabbits inoculated at intervals of 2 weeks, and in some instances of 1 week, when the material used for inoculation was

apparently as favorable in one case as in the other and other conditions under experimental control were common to both sets of animals. On this account, a number of experiments in which the interval between the inoculation of the 2 groups was 1 week or longer have not been included in the present paper. It may be said, however, that the observations derived from these additional experiments are in general agreement with those reported.

The analysis of experimental data may conveniently begin with the first result of inoculation, namely, the primary tumor. It has been our experience with the intratesticular route of injection that a primary tumor rarely fails to develop. In the present series of normal rabbits no failures were observed, but in 2 of the immune animals no tumor appeared. Although this proportion of failures is small, and may be entirely due to an error in the technic of inoculation, it is of interest that they occurred in the immune and not in the normal series. No attempt was made to measure the rate of growth and ultimate size of the primary tumors nor the speed and extent of regression in the instances in which this change occurred, but the general impression obtained of the initial reaction was that the tumors tended to develop more slowly and more irregularly in immune than in normal rabbits.

It will be seen by reference to Table I that in 3 experiments the disease was considerably less severe in rabbits immunized to Virus III than in normal animals; the virus-free strain of the tumor was used in these experiments. The lower level of malignancy in the immune group of each experiment is clearly brought out by the lower mortality rate, the fewer instances of pronounced malignancy, the smaller number of metastatic foci, the lower relative and actual rates of these growths and the higher incidence of actual and probable recoveries. As far as incidence of metastases is concerned, there is no consistent difference in the 3 experiments, but if the data are combined, the incidence is slightly lower among the immune than among the normal rabbits, that is, 48.3 per cent as contrasted with 55.2 per cent.

One experiment is available in which the behavior of the virus-bearing tumor was studied in 10 normal rabbits and in 10 rabbits immunized to Virus III (Table II). Again, the disease was much milder in the immunized group. The mortality rate was only half as

great, and the incidence of well marked cases of malignancy was one-fourth as high as in the group composed of normal animals. There was a much lower number of metastatic foci with a consequent reduction in the relative rate of these growths and although there was no difference in the incidence of metastases, the actual rate, in which only the animals with metastases are considered, was much smaller in the immune group, that is, 6.7 as compared with 16.8 in the normals.

The next comparison has been made with the virus-bearing tumor in normal and the virus-free tumor in immunized rabbits (Table III). The results of the 4 experiments are in general conformity with the others, but the contrast between the level of malignancy displayed by the normal groups and the very mild character of the disease of the immune series is even more pronounced. In each experiment, for instance, there were 3 or 4 cases of outspoken malignancy among the normal groups, but there was only 1 such case among all the immunes of the 4 experiments. There was also a much lower incidence of metastases in 3 immune groups, while in the 4th (Experiment VIII) it was slightly lower. As far as the numbers of metastatic foci and the relative and actual rates of these growths are concerned, the values for the immune groups are uniformly smaller than those for the normals.

In the last experiment, observations of immune rabbits inoculated with virus-bearing tumor are compared with those of normal rabbits inoculated with virus-free tumor (Table IV). The results of this experiment are not in accord with the others. Thus, there was the same incidence of pronounced cases of malignancy and practically the same number of metastatic foci with comparable relative rates of secondary growths in both immune and normal groups. In 2 particulars, however, the disease of the immune group was more severe than that of the normals, namely, in the higher incidence of metastases and in the slightly smaller number of actual and probable recoveries. But the disease of the immune group was considerably less severe than that of a group of normal rabbits inoculated with the same material (Experiment IV, Table II), so that as far as the reaction of the host to this particular inoculum was concerned, the resistance of rabbits immune to Virus III was more effective than that of normal

animals. It is probable, therefore, that the discordant results of the experiment were associated with the other group of animals, namely, the normal rabbits inoculated with virus-free tumor. The disease which developed in these animals was very mild, but not as mild as in a group of immune rabbits inoculated with the same material (Experiment III, Table I). It is likely, therefore, that the particular results obtained in Experiment IX were largely influenced by the character of the virus-free material used for inoculation. In the earlier work with the virus-free tumor the strain was carried in normal rabbits, but later, Virus III immune animals were used because it was feared that a reinfection of the tumor might occur. During the period of these experiments, the same method of monthly transfer of the virus-free strain in immune rabbits and of the virus-bearing stock tumor in normal rabbits was employed, but the fact that the disease in immune animals was comparatively mild suggests that material from such sources might not be as favorable as transplants taken from normal rabbits. That is to say, from the standpoint of the animal factor, conditions tended to favor growth and development of the tumor in the case of the virus-bearing strain, while the reverse obtained with the virus-free strain. On the other hand, it is important to note that the virus-free strain after a sojourn in immunized animals was still capable of inducing a process of well marked malignancy, for there were 2 such occurrences in the normal rabbits of Experiments II and III (Table I). It would appear, therefore, that the comparable results obtained with the immune and normal groups of Experiment IX were due, not to the failure of the immune state as such to be associated with a comparatively mild disease, but to the low level of the malignant process which developed in the normal rabbits.

Finally, as a conclusion to the comparison of individual experiments, the data of 7 experiments (I, II, III, IV, VI, VIII and IX) have been combined. The other 2 experiments cannot be used for this purpose because the data of their immune, although not of their normal groups, appear in other experiments. The observations are derived from 69 normal and 70 immune rabbits and have been analyzed in the same manner as in individual experiments with the following results:

	Total deaths	Malig-nant cases	Metastases			
			Incidence	No. of foci	Relative rate	Actual rate
	per cent	*per cent*	*per cent*		*per cent*	*per cent*
Normals..................	27.53	23.19	56 50	444	6.43	11.37
Immunes.........	10 00	4 29	47 15	174	2.48	5 27

The result of this analysis brings out in a striking manner the fact that the character of the malignant process in rabbits immunized to Virus III was much less severe than in normal rabbits, and further, that this lowered plane of malignancy occurred despite relatively little difference in the incidence of metastases. Virus III immunity did not diminish the number of rabbits in which metastases were found, but the unfavorable effect of this state upon the disease was evidently exerted upon the development of certain of these secondary growths as shown by the total number of foci, together with their relative and actual rates. But this unfavorable effect did not invariably occur, for there were 2 instances of pronounced malignancy in 2 immune groups (Experiments I and IV), a ratio of, roughly, 1 in 10. It is evident, therefore, that the reaction of the exceptional animal is little, if at all, influenced by the presence of an immunity to Virus III, and the inclusion of these rabbits disturbs the tendency toward numerical uniformity of pathological manifestations otherwise obtaining in animal groups in which the tumor process is of low malignancy.

The question of the comparative effectiveness of the immune state of rabbits to Virus III with respect to its duration cannot be properly discussed at this time because of insufficient material. Rabbits injected with Virus III become refractory to subsequent injections of the virus (intradermal) within 6 to 8 days, and the sera of such animals show well marked virucidal properties within a fortnight. As far as is known, these conditions continue for at least 6 months. The present experiments were performed 20 to 39 days after the injection of Virus III when a high state of immunity to the virus was present, but it is impossible to say whether variations in the degree of immunity associated with different periods of duration, if such variations exist, could be satisfactorily demonstrated by means of the malignant disease.

Finally, attention should be drawn to the fact that the immunity to Virus III which follows the injection of the virus-bearing tumor does not appear to be associated with an unfavorable influence upon the malignant process. The time necessary for the development of an immunity under these circumstances may account for this result. In the case of rabbits inoculated with testicular tissue emulsions rich in virus content, the immune state is fully developed within 6 to 8 days, but this period is lengthened to 2 to 3 weeks after the injection of the virus-bearing tumor. This difference in time is presumably due to differences in the amount or state of the virus. Under circumstances of rapid testicular passage at 4 or 5 day intervals, the amount of active virus must be very large, while it is undoubtedly smaller in the case of the tumor transferred at monthly intervals as shown by the fact that although testicular inoculations of stock tumors 4 to 8 weeks old lead to an immunity to Virus III, no visible virus reaction is obtained by means of intracutaneous inoculation of the same material.

Since the findings of the experiments reported in this paper show that the tumor process was not as mild in normal rabbits inoculated with virus-bearing tumor as in comparable groups of rabbits in which a Virus III immune state was present at the time of inoculation, it would appear that the character of the tumor process as a whole was largely determined by conditions or factors obtaining during the first 2 weeks after inoculation. As far as this particular tumor is concerned, however, such an assumption is not entirely warranted. For instance, young primary tumors which have grown slowly and to a limited extent for the first 2 or 3 weeks may suddenly assume an active and rapid growth. It is reasonable to presume that a similar change takes place in any metastatic growths with the result that what was apparently a more or less controlled tumor process became an uncontrolled one. The balance which exists between the ability of the host to control the malignant disease on the one hand, and the capacity of the tumor process toward the continued growth of primary and metastatic tumors on the other, is obviously influenced by a variety of factors. Nevertheless, it would appear from the present experiments that the growth capacities of the virus-bearing transplant were not affected by a slowly developing immunity to Virus III in the same manner as in

the case of an immunity present at the time of inoculation. On the other hand, the failure of the tumor process to be influenced by a delayed virus immunity may be explained upon the basis of the effect which the virus exerts on the animal host. This aspect of the question is discussed in the accompanying paper dealing with the effect of Virus III on the malignant disease (4).

The effect of host immunity to Virus III upon the manifestations of this malignant tumor must be of an entirely non-specific nature. Virus III has been extensively studied in a large number of rabbits for 4 years and there is no indication that it produces tumors of any type. Although the stock tumor with which we have worked was found to be infected with Virus III, there is no reason for assuming that its presence was anything but a fortuitous occurrence and due to 2 factors, first, that this virus is indigenous to rabbits and second, that the tumor presents unusually favorable conditions for the growth and survival of certain viruses. Furthermore, a virus-free strain of the tumor has been found to possess the essential characteristics of malignancy exhibited by the virus-bearing strain, for it can be transplanted from rabbit to rabbit, it gives rise to metastatic growths and it has caused death. The effect of Virus III immunity upon the course and character of the neoplasm has, moreover, been observed in the disease induced by both virus-free and virus-bearing tumors. And it may be mentioned in this connection that similar effects have been observed in connection with concomitant infections which have affected the course of the tumor and also experimental infection with *Treponema pallidum.**

The mechanism by which this effect is produced is not known. If one considers resistance or susceptibility to disease as a functional activity of the animal organism, then it is evident that the low plane of malignancy displayed by rabbits immunized to Virus III was brought about by factors which affected animal economy resulting in an increased or a more effective resistance to the tumor process.

SUMMARY AND CONCLUSIONS.

Experiments are reported in which were studied the course and character of a transplantable malignant neoplasm in normal rabbits and in rabbits immunized with a filterable virus, Virus III.

* Unpublished experiments of L. Pearce.

The disease which developed in immunized rabbits was extremely mild and much less severe than in normal animals.

The effect upon the tumor process displayed by Virus III immune rabbits in the direction of diminished malignancy is considered to be entirely non-specific in character, and the suggestion is made that it is accomplished through a more effective resistance of the host.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1927,
Vol. xlvi, No. 1, pp. 81–99.]

EFFECT OF A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

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(Received for publication, March 22, 1927.)

In connection with the study of the effect of an immunity to Virus III on the course of a transplantable neoplasm of the rabbit (1), other problems relating to the tumor were investigated. The finding that the stock tumor (2) was infected with Virus III (3) which was being regularly transferred with it, suggested the question whether the presence of Virus III affected the growth and malignancy of the tumor. The fact that we were able to obtain the tumor without the virus and to transfer it from rabbit to rabbit furnished the means for an experimental study of this problem, the results of which are reported in the present paper.

EXPERIMENTAL.

Materials and Method.

The general plan of the experiments consisted of the study of parallel series of rabbits inoculated with the virus-bearing and the virus-free strains of the tumor. The dates of inoculation were as follows:

Experiment I	January 14 and 17, 1925.
Experiment II	February 16, 1925.
Experiment III	March 13 and 18, 1925.
Experiment IV	April 15 and 17, 1925.
Experiment V	October 27 and 28, 1925.
Experiment VI	November 24 and 25, 1925.
Experiment VII	November 24 and 25, 1925.

Neoplasm.—The tumor which is described fully in earlier papers (2) is considered to be of epithelial origin.

Virus III.—This has been described in the preceding paper (1) and more detailed accounts have previously been published (3).

TABLE I.

Comparison of Virus-Bearing and Virus-Free Tumor in Normal Rabbits.

Experiment	Group	No of rabbits	Primary tumors	Mortality						Metastases					No of metastatic foci			Probable deaths	Actual and probable recoveries	
				Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors				
				No	Per cent	No.	Per cent	No	Per cent	No.	Per cent								No.	Per cent
I	V +	10	All +	2	20 0	0	—	2	20 0	4	40 0	26	2 6	6.5	10, 7	5, 4	0 in 6	1	7	70.0
	V −	9	All +	6	66 6	2	22.2	4	44 4	9	100 0	61	6 8	6 8	15,* 11, 9, 7, 5, 3	8, 2, 1	0 in 0	1	2	22.2
II	V +	9	All +	4	44 4	4	44 4	0	—	5	55 5	81	9 0	16.2	30, 17, 15, 15	4	0 in 4	0	5	55.5
	V −	9	All +	6	66 6	2	22 2	4	44 4	6	66 6	70	7 7	11 7	22, 15, 10, 9, 9, 5	—	0 in 3	0	3	33.3
III	V +	10	All +	5	50 0	4	40.0	1	10 0	7	70 0	74	7 4	10 6	21, 17, 16, 9, 7	3, 1	0 in 3	0	5	50.0
	V −	9	All +	3	33 3	2	22 2	1	11 1	6	66 6	62	6.9	10 3	22,* 20, 14	3, 2, 1	0 in 3	1	5	55.5
IV	V +	10	All +	2	20 0	0	—	2	20 0	4	40 0	26	2 6	6 5	12, 8	4, 2	0 in 6	2	6	60.0
	V −	10	9 +	3	30 0	2	20 0	1	10 0	8	80 0	63	6 3	7 9	19,* 14, 12	7, 5, 4, 1, 1	0 in 2	2	5	50.0
V	V +	11	All +	4	36.4	3	27.3	1	9 1	9	81.9	74	6 7	8.2	26, 19, 14, 6†	4, 2, 1, 1, 1	0 in 2	0	7	63.6
	V −	10	9 +	1	10.0	1	10 0	0	—	6	60 0	41	4 1	6 8	25	4, 4, 3, 3, 2	0 in 4	1	8	80.0
VI	V +	10	All +	4	40 0	4	40 0	0	—	6	60 0	101	10 1	16 8	32, 30, 16, 12	9, 2	0 in 4	1	5	50.0
	V −	10	All +	1	10 0	1	10.0	0	—	4	40 0	32	3 2	8 0	21	8, 2, 1	0 in 6	1	8	80.0
Total	V +	60	All +	21	35 0	15	25 0	6	10 0	35	58 3	382	6 48	10.92				4	35	58.3
	V −	57	55 +	20	35 1	10	17 6	10	17 6	39	68 4	329	5 78	8 44				6	31	54.4

TABLE I.—*Concluded.*

Comparison of Virus-Bearing and Virus-Free Tumor in Virus III Immune Rabbits.

Experiment	Group	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci			Probable deaths	Actual and probable recoveries	
				Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors				
				No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								No.	Per cent
VII	V +	10	All +	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	6.7	25, 6	4, 2, 2, 1	0 in 4	0	8	80.0
	V −	11	All +	0	—	0	—	0	—	6	55.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	11	100.0
Total of all experiments	V +	70	All +	23	32.9	16	22.9	7	10.0	41	55.9	422	6.2	10.3				4	43	61.4
	V −	68	66 +	20	29.4	10	14.7	10	14.7	45	66.2	349	5.1	7.8				6	42	61.8

V+ = virus-bearing tumor, V− = virus-free tumor.

* Number of metatastic foci estimated.

† Complicating empyema.

TABLE II.

Analysis of Metastatic Foci in All Fatal Cases.

Group	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.
Virus-bearing				I 7	I 10
tumor	II 30*	II 15*			
	II 17*				
	II 15*			III 17*	
		III 21*		III 9*	
		III 16*		III 7	
	IV 12				
	IV 8			V 14*	
	V 26*	V 19*	V 6	VI 12*	
	VI 30*	VI 32*			
		VI 16*			
		VII 6	VII 25*		
Virus-free	I 5	I 3	I 7	I 9	I 1†*
tumor			I 15*†		
	II 9	II 22*	II 15*	II 9	
	II 5	II 10			
	III 14	III 22*†			
		III 20*			
			IV 12	IV 19*†	14*
		V 25*			
	VI 21*				

Deaths 4th and 5th wks.	Malignant cases		Total foci	Foci in malignant cases		Deaths 6th, 7th and 8th wks	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent		No.	Per cent		No.	Per cent
V + 14	11	78 6	263	237	90.1	V + 9	5	55.5	107	77	72.0
V − 11	5	45.5	156	110	70.5	V − 9	5	55.5	111	74	66.8

Total deaths	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent
V + 23	16	69 6	370	314	84 9
V − 20	10	50.0	267	173	64 8

Roman numerals refer to number of experiment; Arabic to number of metastatic foci.

* Instances of well marked malignancy.

† Number of foci estimated.

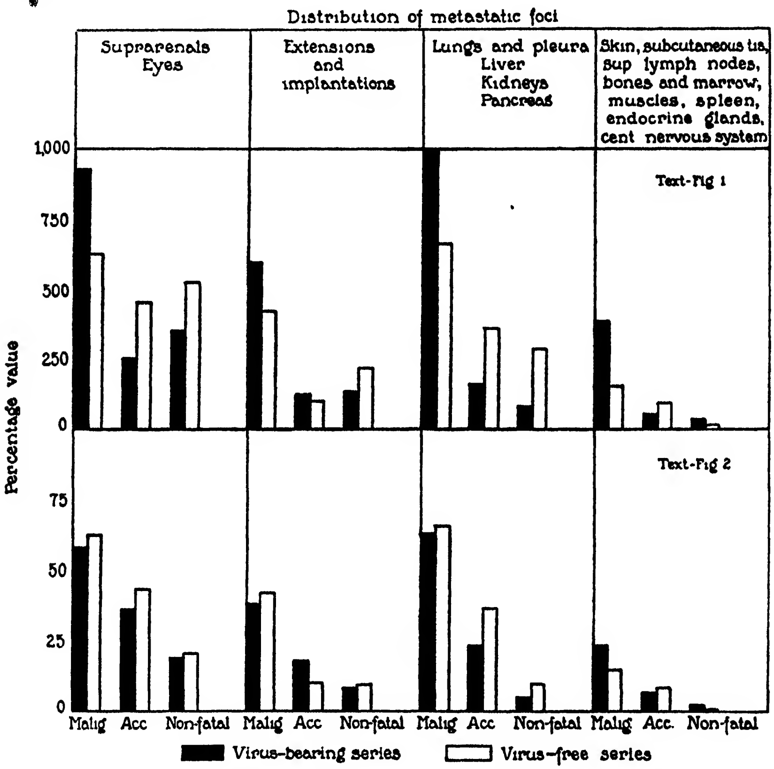
TABLE III.

Distribution of Metastatic Foci.

Group	No. of rabbits	I. Suprarenals and eyes				II Extensions and implantations				III. Lungs				IV. Skin, subcut. tissue, super lymph nodes, etc.				Total foci	
		No of foci	Per cent	Foci per rabbit	Per cent	No of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No of foci	Per cent	Foci per rabbit	Per cent	No.	Per rabbit
Malignant cases																			
V +	16	37	925.0	2 31	57 8	112	593 6	7 0	37 1	50	1000 0	3 12	62 5	115	379 5	7 2	23 7	314	19.6
V −	10	25	625 0	2 5	62 5	79	418 7	7 9	41 9	33	660 0	3 3	66 0	47	155 1	4 7	15 5	184	18 4
Accidental deaths																			
V +	7	10	250 0	1 43	35 7	23	121 9	3 3	17 4	8	160 0	1 42	22 9	15	50 0	2 1	7.1	56	8 0
V −	10	17	425 0	1 7	42.5	20	106 0	2 0	10 6	18	360.0	1 8	36 0	28	92 4	2 8	9 2	83	8 3
Non-fatal cases																			
V +	18	14	350 0	0 78	19 4	25	132 5	1 4	7 4	4	80 0	0 2	4.4	9	29 7	0 5	1.7	52	2 9
V −	25	21	525 0	0 84	21 0	41	217 3	1 6	8.7	14	280 0	0 6	11 2	6	19 8	0 2	0 8	82	3.3
Total																			
V +	41	61	1525 0	1.48	37 2	160	848 0	3 9	20 7	62	1240 0	1 5	30 2	139	458 7	3 4	11 2	422	10.3
V −	45	63	1575 0	1 4	35 0	140	742 0	3 1	16 5	65	1300 0	1 4	28.9	81	267 3	1 8	5.9	349	7.8

Virus-Free Strain of the Neoplasm.—This has been described in an earlier report (4) and in the preceding paper (1).

The experiments reported in this paper were carried out in the same manner as those previously reported (1).

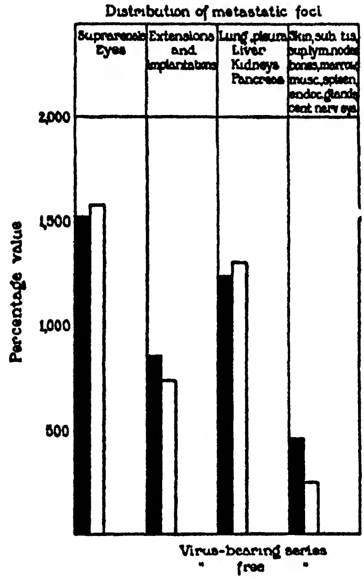


TEXT-FIG. 1. Distribution of metastatic foci in different types of disease.

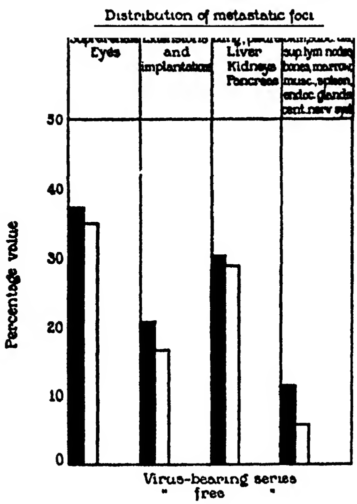
TEXT-FIG. 2 Average distribution per animal of metastatic foci in different types of disease.

Method of Analysis of Results.—Clinical and postmortem observations have been analyzed in the same way as those given in the preceding paper (1). The secondary growths have also been considered in relation to the organs and tissues involved.

The percentage estimations of metastatic distribution have been based upon the number of foci theoretically possible as shown by the actual location of metas-



TEXT-FIG. 3. Distribution of metastatic foci in all cases.



TEXT-FIG. 4. Average distribution per animal of metastatic foci in all cases.

tases in the first 20 generations of tumor animals (5). Certain obvious objections may be made to this method. Such organs as the liver or kidneys, for example, may show numerous tumors or only a few which destroy little of the organ. But, the general character of the disease, whether of high, moderate or low malignancy, is shown by grouping the metastatic foci in the following divisions:

I.	Suprarenals and eyes...	4 possible foci
II.	Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum...	19 " "
III.	Lungs and pleura, liver, kidneys and pancreas. ..	5 " "
IV.	Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system.....	30 " "

This arrangement which has been employed in analyzing the results of other tumor experiments (6) was selected upon the basis of observations of several hundred rabbits inoculated with this tumor. Suffice it to say that in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation, there is usually a widespread distribution of secondary growths to many organs and tissues, including the skin, muscles, bones and bone marrow and the endocrine glands which are practically never involved in cases of low malignancy. In those animals in which the disease is very mild, metastases may be found only in the eyes or suprarenal glands or in the facial and jaw bones and cranial sinuses which also, of course, are frequently involved in instances of a severe disease. All gradations are found between these extremes of malignancy.

Results.

The results of the 7 experiments here reported consist of the clinical and postmortem observations of 138 rabbits, 70 of which were inoculated with the virus-bearing and 68 with the virus-free strain of the tumor. For convenience of discussion, these observations are summarized in Table I.

An analysis of the metastatic foci found in all fatal cases is based upon the time at which such deaths occurred with reference to the time of inoculation (Table II).

A further analysis of the character of the disease takes into account the various organs and tissues actually involved by metastases (Table III). The charts of Text-figs. 1 to 4 are graphic representations of the numerical values of Table III.

DISCUSSION.

The experiments reported were undertaken for the purpose of ascertaining whether the presence of Virus III in a transplantable neoplasm of the rabbit influenced the character of the malignant disease. Because of the variability of the tumor process which may be influenced by many factors, the value that can be attached to differences in disease manifestations must be interpreted with due consideration of the circumstances of the experiment. In the present study the factors which could be controlled were common to both animal groups of each experiment.

The most important variable which could not be controlled was the inoculating material itself which, of course, was derived from different sources and there was no means of ascertaining whether there were actual or potential differences of growth capacity in the 2 tissues. It might be said that this difference in material constitutes an objection to the comparison of results, but since the experiments necessitated the presence of the virus in one strain of the tumor and its absence in the other, the use of transplants from different sources was unavoidable. In both strains the tumor employed for transfer was carefully selected with the intention of providing as favorable material in the one case as in the other. With the virus-bearing strain (the stock tumor) transfers were regularly made at monthly intervals as had been our custom for several years. This method was also followed with the virus-free strain during the time of these experiments, but immediately preceding the first experiment, transfers had been made more frequently which may have influenced the results obtained. The material used in the last 3 experiments was obtained from rabbits which had been immunized to Virus III, and as has been pointed out in the preceding paper (1), there is some evidence to show that material from such a source is not as favorable as that obtained from normal rabbits. This feature will subsequently be discussed more fully, but it may be stated now that material subjected to such supposedly deleterious influences as freezing, thawing and grinding in the frozen state before being used for inoculation appeared to have no appreciable effect on the character of the disease produced (7). While these procedures were extraneous as contrasted with possible effects of the virus

within the tumor, both being subjected to the influence of the animal organism, the results of the experiments referred to show that chance inequalities of material used for inoculation may not be a factor of any considerable importance where extreme variations of malignancy are concerned.

The first impression obtained from the summary of the clinical and postmortem observations in Table I is the variability of the disease picture in both the virus-bearing and the virus-free groups. This feature was, with 2 exceptions, more pronounced in the case of the virus-bearing strain, as shown by percentage variation values of various disease manifestations. In the case of the total mortality rate, the virus-free series was the more variable while the percentage variation of metastatic incidence was the same in both series.

Virus-bearing tumor						Virus-free tumor					
Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate	Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate
per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*
50 0	44 4	81 9	101	10 1	16 8	66 6	22.2	100 0	70	7 7	11 7
44 4	40 0	70 0	81	9 0	16 2	66 3	22.2	80 0	63	6.9	10 3
40 0	40 0	60 0	74	7 4	8 2	33 3	22 2	66 6	62	6 8	8 0
36 0	27 3	60 0	74	6 7	6 9	30.0	20.0	66.6	61	6 3	7 8
20 0	10 0	55 5	40	4 0	6 7	10 0	10.0	60 0	41	4 1	6 8
20 0	0	40 0	26	2 6	6 5	10 0	10 0	55 5	32	3 2	6 8
20 0	0	40 0	26	2 6	6 5	0	0	40.0	20	1 8	3 3
Percentage variation 33.7	73 3	19 2	42 1	42 2	36 3	68 1	48 0	19 7	32.4	36 3	24 4

If the results of all experiments are combined, the disease produced by the virus-bearing strain appears to be more severe (Table I). The number of cases of well marked malignancy was distinctly greater, 22.9 as contrasted with 14.7 per cent; there were more foci of metastases, 422 as compared with 349, and in addition, the relative and actual metastatic rates were higher, 6.2 and 10.3 as contrasted with 5.1 and 7.8 respectively. The incidence of metastases, however, was slightly lower, 55.9 as contrasted with 66.2 per cent. There was no

difference in the proportions of actual and probable recoveries, 61.0 per cent of each series falling in this category and little difference in the small numbers of probable deaths, so that the observations of the fatal cases are of particular importance.

In the analysis which has been made of the fatal cases (Table II), special attention is given to the time of death since the majority of rabbits with a process of pronounced malignancy die within 3 to 5 weeks after inoculation. Reference to Table II shows that approximately the same proportion of deaths in each group occurred within the 4th and 5th weeks after inoculation, that is, in the case of the virus-bearing series 14 out of 23 cases or 60.9 per cent and 11 out of 20 or 55.0 per cent in the virus-free series. But there was a decided difference in the incidence of cases of well marked malignancy, for 11 of the 14 deaths (78.6 per cent) in the virus-bearing series were of this type as contrasted with 5 of the 11 deaths (45.5 per cent) in the virus-free group. The proportion of metastatic foci in these malignant cases to the total number of foci found in all fatal cases of the 4th and 5th weeks shows a striking difference, that is, virus-bearing, 90.1 per cent; virus-free, 70.5 per cent. When the deaths which occurred in the last 3 weeks of the observation period are similarly considered, however, there is little difference between the 2 groups.

If these comparisons be made with all deaths irrespective of time, the values obtained as given below would indicate that the more severe condition developed in the disease produced by the virus-bearing tumor.

Group	Total deaths	Malignant cases		Total metastatic foci	Foci in malignant cases	
		No	Per cent		No	Per cent
Virus-bearing	23	16	69 6	370	314	84 9
Virus-free..	20	10	50 0	267	173	64 8

The higher incidence of cases of well marked malignancy in the virus-bearing series is of considerable significance in view of the total number of animals comprising each group (Table I). There was practically no difference in the total mortality rates of the 2 series, however, nor in the time distribution of all deaths, but there was a considerable

difference in the incidence and time of the so called accidental deaths as shown by the following data:

Group	No of rabbits	Total deaths		Accidental deaths		Deaths 4th and 5th wks.		
						Total	Accidental	
		No	Per cent	No.	Per cent		No.	Per cent
Virus-bearing..............	70	23	32 9	7	30 4	14	3	21 4
Virus-free.............	68	20	29 4	10	50 0	11	6	54.5

The accidental deaths are directly caused by the particular location of a metastasis such as the spine, and other secondary growths are not necessarily numerous or destructive, especially if death occurs within the first few weeks. These cases cannot be dismissed as entirely lacking in malignant potentialities, however, for it is not impossible that at least some of them would have subsequently died from a more extensive tumor process had the immediate cause of an early death not occurred. But, as far as the average numerical distribution of foci in the different types of fatal cases is concerned, the following figures show little difference between the virus-bearing and the virus-free series:

Deaths Due to Well Marked Malignancy.

Group	4th and 5th wks: No of rabbits	4th and 5th wks: No of metastatic foci	4th and 5th wks: Rate	6th, 7th and 8th wks: No of rabbits	6th, 7th and 8th wks: No. of metastatic foci	6th, 7th and 8th wks: Rate
			per cent			*per cent*
Virus-bearing.........	11	237	21 5	5	77	15.4
Virus-free..........	5	110	22 0	5	74	14 8
Accidental Deaths.						
Virus-bearing..............	3	26	8 7	4	30	7.5
Virus-free.............	6	46	7 7	4	37	9.3

As far as the analysis of results has been carried, the chief points of difference between the 2 series of rabbits are (1) the higher incidence

of fatal cases with a tumor process severe enough to be classified as "malignant" in the virus-bearing series, and (2) a lower incidence of "malignant" cases but a higher incidence of early "accidental" deaths in the virus-free series.

The question at once arises as to how much importance should be attached to such points of difference as indicating variations of disease severity which could be ascribed, either directly or indirectly, to the presence or absence of Virus III in the tumor. Before this subject is discussed, the character of the disease should be considered from the standpoint of the organs and tissues involved. In such an analysis it is convenient to adopt some such plan as was outlined in the section on Materials and Method which consists in grouping the secondary tumors observed at postmortem examination into major divisions corresponding to the distribution of metastases which have been found in association with tumor processes of various degrees of severity (Table III).

The first column of each of the 4 divisions of Table III contains the total number of foci found while the figures in the second column represent the average number of foci per animal. The percentage values have been calculated upon the basis of the total number of theoretically possible growths in these sites. Text-figs. 1 to 4 are graphic representations of the values of Table III. The analysis includes all cases in which secondary growths were found and the classification of these cases previously employed has been followed, that is, malignant cases, accidental deaths and animals surviving the observation period.

Reference to Table III and to Text-fig. 1 shows that as far as the malignant cases are concerned, there were many more metastatic foci in all 4 divisions of secondary growths in the virus-bearing than in the virus-free series, which is to be expected in view of the larger number of these cases in this group. When the average value per animal is considered, however, there is little difference between the 2 series except in one important division (IV), namely, that which includes the skin, muscles, bones and endocrine glands (Text-fig. 2). Metastases to these tissues and organs rarely occur except in cases of well marked malignancy. It must be remembered, moreover, that the number of theoretically possible foci in this division is much larger than in the others, so that small differences are significant, as in the present in-

stance in which the values for the virus-bearing and virus-free series were 7.2 and 4.7 per rabbit, respectively. The distribution of secondary growths would indicate, therefore, that the tumor process in the cases of well marked malignancy was more severe in the virus-bearing than in the virus-free series.

In the group of accidental deaths, on the other hand, the disease was slightly but definitely more severe in the virus-free group (Table III; Text-figs. 1 and 2). The values for this group in the 1st, 3rd and 4th divisions of metastatic foci are all larger than those of the virus-bearing series. In the 2nd division, that of extensions and implantations, the figures for the virus-bearing series are the larger; but as has been pointed out, tumors in this category as well as those in the suprarenal glands and the eyes (Division I) possess far less significance from the standpoint of disease severity than do those in such locations as the lungs, liver, kidneys, skin, muscles, bones and other glands of internal secretion (Divisions III and IV). The reason for the higher incidence of accidental deaths in the virus-free series is not clear, but the fact that the disease in these cases was more severe in the virus-free than in the virus-bearing group suggests that under other circumstances, such as a larger number of animals or another experimental period, the malignant level of the entire virus-free series might more nearly approach that of the virus-bearing series.

If the figures for these disease types—the malignant cases, the accidental deaths and the non-fatal cases—are combined and analyzed in the same manner (Table III; Text-figs. 3 and 4), it is seen that there was practically no difference between the virus-bearing and the virus-free groups in regard to extensions and implantations (Division II), and to the distribution of metastases to the suprarenals and eyes (Division I), and to the lungs, liver and kidneys (Division III). On the other hand, the more frequent involvement of the skin, subcutaneous tissues, muscles, bones, heart, central nervous system and glands of internal secretion (Division IV) in the virus-bearing series points to a tumor process of a somewhat higher malignancy than that of the virus-free series. The conclusion from these analyses is in accord with those previously arrived at from a consideration of such features of the disease as the mortality rate, the type of fatal case and the number and rate of metastatic foci.

The combined observations of all experiments have so far been considered but if the results of individual experiments are compared, it is seen that they are not entirely constant (Table I). The degree of malignancy in Experiments II, III, V, VI and VII was greater in the virus-bearing groups as shown by the incidence of malignant cases, the total number of metastatic foci and the relative and actual rates of the growths. But in Experiments I and IV, the disease of the virus-free groups was more severe. In these 2 experiments the tumor process of the virus-bearing series was very mild, much more so, in fact, than in any other group in any experiment except the last, No. VII, in which the disease of both groups was benign. Rabbits immunized to Virus III were employed in this experiment, which condition is associated with a disease of low malignancy (1).*

Pronounced variations in malignancy in groups of rabbits inoculated with the stock tumor at consecutive monthly intervals have been observed over long periods. From an analysis of the first 20 generations of the tumor, it appeared that the principal factors concerned in determining the results of transplantation were adaptation to passage and variations in meteorological conditions that prevailed during the time the experiments were carried out,—the one affecting the energy of cell growth, and the other affecting animal economy (7). It must be assumed that meteorological influences would be exerted over both groups of animals in each of the present experiments. On the other hand, the possibility that such an agent as Virus III might affect the energy of cell growth should be considered. The filterable viruses are intimately associated with cells, and frequently with young cells in particular, and it is possible that with the tumor some biological effect would be induced by the presence of Virus III, perhaps of the nature of a stimulation to cell growth. In the case of vaccine virus and Virus III, for example, the idea of cell stimulation, probably in connection with cell injury, is supported by the histological picture of early lesions of the cornea and skin of rabbits.

* In Experiment VII, the disease was not as mild in the virus-bearing as in the virus-free group. It is of interest to note that of the 20 metastatic foci in the virus-free group (Table I), 1 was healed and 13 were largely or wholly necrotic. In the virus-bearing group, on the other hand, a similar condition was noted in but 3 of the 40 metastatic foci.

The growth capacity of the tumor cells may be so great that the stimulation to cell growth induced by an agent like Virus III would be entirely negligible. On the other hand, if such an influence is especially exerted upon young cells, and if it results in an increase in the rate of cell multiplication, a more vigorous growth of the early primary tumor would probably take place, which might lead to the production of a more severe disease. However, the tumor process is affected by a variety of factors and conditions, some of which might favor and others oppose the hypothetical influence of Virus III and the mildness of the disease in Experiments I and IV may be examples of the successful opposition, from an unknown cause, of the more usual effect associated with the virus.

Attention should also be called to the fact that although the disease of the virus-free groups was more uniform than that of the virus-bearing groups with respect to certain pathological manifestations, there was a definite tendency toward a lower plane of malignancy in the last as compared with the first experiments. During the course of this study, both strains of the tumor were transferred at monthly intervals and as presumably favorable material was used in the one case as in the other. But immediately preceding the experiments, the virus-free strain had been transferred more frequently, and although no conclusive evidence is available that such a procedure promotes increased malignancy, other factors being equal, still the possibility should be considered. During the latter part of this work, the virus-free tumor was carried in rabbits which had been immunized to Virus III, and observations show that in them there is a definite tendency toward a disease of lowered malignancy (1). It is possible, therefore, that the inoculating material derived from Virus III immune animals was of a less favorable character and as such may have contributed to the decreased severity of the disease as it appeared in the virus-free groups of the last 3 experiments.

Among other factors which may have indirectly influenced the course of the process induced by the virus-bearing tumor, there is one in particular which merits special attention, namely, the effect of Virus III on the rabbit host. It will be recalled that a certain number of normal rabbits, 15.0 to 20.0 per cent on an average in this laboratory, have been found to be immune to Virus III. It has also been observed

that the tumor process tends to be less severe in rabbits which have been experimentally immunized to Virus III than in normal rabbits (1). From these facts, it would appear that the chance inclusion of one or more Virus III immune rabbits in a group of 10 animals would affect the experimental results in the direction of diminished malignancy. There was just as much chance, however, for immune rabbits to be included in the virus-bearing as in the virus-free groups. All rabbits inoculated with the virus-free tumor which were subsequently tested for an immunity to Virus III showed a typical cutaneous reaction, indicating the absence of an immunity. Preliminary virucidal tests of the sera of rabbits subsequently inoculated with the virus-bearing tumor were not carried out in the present series of experiments, so that it is impossible to say whether any Virus III immune animals were included in these groups.

On the other hand, one must take into account the possible effect of Virus III upon the course of the disease from the standpoint of the more immediate reaction of the animal host to the introduction of the virus with the tumor. At first glance, one might suppose that the severity of the tumor process would be diminished since the general plane of malignancy tends to be low in rabbits immunized to Virus III and rabbits inoculated with the virus-bearing tumor develop an immunity to the virus. It has been shown, however, that such is not the case, the probable explanation being that the immunity occasioned by the virus-bearing tumor develops more slowly than that intentionally caused by the injection of tissue emulsions rich in virus content (1). When an immunity is fully developed in connection with virus-bearing transplants, the tumor process has apparently reached the point when it is not affected by the immune state of the host, to the degree, at least, that is observed in rabbits which are already immune to Virus III at the time of tumor inoculation. On the other hand, the presence of the virus within the tumor affects the animal host since an immunity to it eventually develops, and it is not unlikely that during the development of this state, the response of the host to other pathogenic agents would be affected. Thus, in the present instance, the somewhat greater severity of the disease induced by the virus-bearing strain may be due to an increased susceptibility or a decreased resistance of the host as a collateral or indirect result of the

Virus III infection of the tumor transplant. In Experiments I and IV, in which the disease was unusually mild, it is not unreasonable to assume that there were present factors or conditions which operated to prevent this alteration in the reaction of the host to the tumor or which effectively opposed the result of such an alteration. An unusually low content or diminished activity of Virus III in the material used for inoculation or an already existing immunity to Virus III are factors which might operate in this manner.

The reciprocal effects of concomitant or superimposed disease conditions have received considerable attention, chiefly, however, from a clinical standpoint. That the subject is open to experimental investigation is shown by the results of the present study which suggest that the presence of one pathogenic agent, a filterable virus, is associated with certain variations in the disease induced by a second agent, the malignant tumor. That these variations are not of larger magnitude may be related to the virulence of the virus which is not high as ordinarily considered in terms of the usual criteria of animal reaction, so that a pronounced effect would hardly be expected.

In conclusion, it should be pointed out that the influence of an associated infection with Virus III as a factor concerned in determining variations in growth and malignancy of the tumor must be evaluated with due regard to the many other factors which influence the disease process. There is no indication, for instance, that extreme variations in malignancy could be accounted for on this basis, or that the orderly succession of periods of increasing and diminishing malignancy with a distinct and characteristic tendency to a seasonal distribution are affected by the presence or absence of Virus III in the tumor. The present observations, however, indicate that the presence of Virus III is usually associated with a higher degree of malignancy while the effect of an immunity to Virus III has been shown to be associated with a disease of diminished severity (1). These conditions probably account for some of the irregularities in results observed in individual animals and in certain groups of animals.

SUMMARY AND CONCLUSIONS.

A study of a malignant disease in rabbits has been made with reference to the presence or absence of a filterable virus, Virus III, in the

tumor. The results are analyzed from the standpoint of certain characteristic features of the tumor process in order to determine any differences in degrees of malignancy.

It was found that a more severe disease developed in the series in which the virus-bearing tumor was used than in the series in which the tumor was free of the virus, although the differences were not very marked and were not entirely constant.

The influence of Virus III as a factor affecting malignancy has been discussed from the standpoint of its possible effect upon (*a*) the tumor cells and (*b*) the host reaction. It has been suggested that the greater malignancy of the pathological process usually induced by the virus-bearing tumor is attributable to a change in the response of the host to the tumor, which change is of the nature of a decreased resistance associated with the reaction of the host to the virus infection.

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[Reprinted from The Journal of Experimental Medicine, August 1, 1927, Vol. xlvi, No. 2, pp. 205–211.]

FURTHER OBSERVATIONS ON THE INABILITY TO TRANSMIT A RABBIT NEOPLASM BY CELL-FREE MATERIALS.

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(Received for publication, April 21, 1927.)

The study of a malignant neoplasm of the rabbit which has been in progress in these laboratories for several years, included experiments in 1921 and 1922 in which it was found that propagation could not be accomplished by the use of filtrates of the tumor or of desiccated tumor tissue (1).

As serial transplantation progressed, adaptation to passage has been associated with an increase in the energy of cell growth as shown by alterations in the character of growth of the primary tumor and metastases which have resulted in a more rapid progress of the disease toward death or recovery (2). Under these circumstances, a repetition of the filtration and desiccation experiments was desirable for the purpose of checking the earlier results with material of a more favorable character as far as capacity for growth is concerned. A few experiments were also carried out with fluid media which had been in contact with tumor tissue and subsequently filtered or centrifuged in order to ascertain whether the tumor could be propagated by a cell-free agent obtained by diffusion. The present paper contains the results of these several experiments in the order named.

EXPERIMENTAL.

Materials and Method.

Tumors of the 56th, 65th and 68th generations were used in this study which was carried out in April and October, 1926, and in January, 1927. The material employed was obtained from primary testicular tumors, the growth activity of which was controlled by

intratesticular inoculations of fresh cell emulsions not subjected to any manipulation other than emulsification with normal saline; sand was not used. This procedure and route of inoculation are those used in the majority of experiments with this tumor. In 2 preliminary experiments with Hartley's broth, as described below, a control inoculation of the fresh tumor was not made; the condition of the tissue was such, however, that its transplantation as ordinarily carried out would undoubtedly have resulted in tumor growth.

Filtration —Immediately after removal of the tumor, portions of it were pulped, pressed through a fine meshed sieve and ground with sand. Ringer's solution was added in the proportion of 50 cc. to approximately 5 gm. of tumor. The mixture was shaken for 20 minutes, centrifuged for 10 minutes at a speed of 1500 revolutions per minute and the supernatant fluid filtered through Berkefeld V candles. The cell-free state of the filtrate was controlled by the addition of a suspension of *B. prodigiosus* to the fluid prior to filtration.

A thin suspension of Kieselguhr was thoroughly mixed with the filtrate in the proportion of 0.5 or 1.0 cc. to 25 cc. of the filtrate and 1.0 cc. of the mixture was injected intratesticularly in normal rabbits.

Desiccation.—Small pieces of the tumor were pressed through a fine sieve and a thin layer of the pulp was spread on the bottom of large Petri dishes The dishes were placed over concentrated sulfuric acid in desiccator jars and the air evacuated by a Geryk pump to a pressure of 3 mm. The jars were then kept in a freezing box at a temperatue of -1°C. for 5 days. The material was pulverized and taken up in a small amount of normal saline or of Ringer's solution; 1.0 cc. of this suspension was injected intratesticularly in normal rabbits.

Supernatant Fluid of Tumor "Cultures."—Cubes of tumor tissue measuring approximately 0.5 cm. along each side were placed in test-tubes containing 5 cc. of Hartley's KCl glucose broth and 1 cc. of fresh rabbit serum. The tubes were put in jars from which the air was evacuated, and the jars were kept in the ice box for 48 hours. At the end of this time, the supernatant fluid was centrifuged for 10 minutes at a speed of 1500 revolutions per minute, and filtered through a Berkefeld V candle, *B. prodigiosus* having been added before filtration Inoculations of the filtrate were made into 1 or both testicles of normal rabbits, 0.5 cc. or 1.0 cc. being used, and in one experiment 0.2 cc. was also injected intracutaneously on the ventral surface of the sheath.

In one experiment, the supernatant fluid of the cultures was centrifuged twice but not filtered. Both intratesticular and intracutaneous injections were carried out with this material.

Tissue of "Cultures."—Tumor tissue which had been kept in Hartley's broth in the ice box for 48 hours was emulsified with normal saline and 0.4 cc. of the emulsion was injected into the testicles of normal rabbits.

"Stored" Tissue.—Pieces of the same tumor used for the above culture experiment were placed in small Petri dishes with bits of gauze soaked in normal saline, care being taken that the tissue did not come in contact with the gauze. The dishes were sealed with adhesive tape and placed in the ice box for 48 hours. Each piece of tumor was then emulsified with normal saline and 0.4 cc. of each emulsion was injected into both testicles of 5 normal rabbits.

TABLE I.

Results of Filtration Experiments.

Experiment	Generation of tumor	Tumor filtrate						Controls—fresh tumor					
		No of rabbits	Inoculation			Growth		No of rabbits	Inoculation			Growth	
			Route	Number	Amount	Positive	Negative		Route	Number	Amount	Positive	Negative
					cc						*cc.*		
I	65	10	R. testicle	10	1 0	0	10	10	R. testicle	10	0.3	10	0
			L. "	5	1 0	0	5						
II	68	5	R. "	5	1 0	0	5	5	" "	5	0.3	5	0
			L. "	5	1 0	0	5						

TABLE II.

Results of Desiccation Experiments.

Experiment	Generation of tumor	Desiccated tumor							Controls—fresh tumor					
		No of rabbits	Duration of desiccation	Inoculation			Growth		No of rabbits	Inoculation			Growth	
				Route	Number	Amount	Positive	Negative		Route	Number	Amount	Positive	Negative
			days			*cc.*						*cc*		
I	65	5	5	R. testicle	5	1 0	0	5	10	R. testicle	10	0 3	10	0
II	68	5	5	" "	5	1 0	0	5	5	" "	5	0 3	5	0
				L. "	5	1 0	0	5						

The rabbits were examined frequently in order to determine any reaction at the site of inoculation which could be diagnosed as tumor growth by inspection or palpation. The period of observation varied from 5 weeks to 3 months. With the intratesticular route of inoculation, the usual incubation period of this tumor at present is 5 to 8 days, at the end of which time there is no doubt of the active character of the growth.

In the experiments in which tumor filtrates or desiccates were employed, the rabbits were observed for 2 and 3 months, since it was probable that growth, if any occurred, would be greatly delayed. In the experiment in which centrifuged

TABLE III.

Results of Diffusion Experiments.
Anaerobic Cultures in Hartley's Broth 48 Hours—Ice Box Temperature.

Experiment	Generation of tumor	Supernatant fluid	Inoculation						Controls—fresh tumor					
			No of rabbits	Site	Number	Amount	Growth		No of rabbits	Site	Number	Amount	Growth	
							Positive	Negative					Positive	Negative
						cc						cc		
I	56	Centrifuged	5	Testicle	5	0 5 in 3 1 0 " 2	0	5						
				Sheath	5	0 2	0	5						
II	56	" and filtered	5	Testicle	5	0.5 in 3 1 0 " 2	0 0	5						
				Sheath	5	0 2	0	5						
III	68	Centrifuged and filtered	5	Testicle	10	1 0	0	10	5	Testicle	5	0 3	5	0

TABLE IV.

Results of Experiments with Stored Tissue.

Procedure		Inoculation				Growth	
		No of rabbits	Site	Number	Amount	Positive	Negative
					cc.		
Hartley's broth (anaerobic)	ice box 48 hrs.	5	Testicle	10	0.4	0	10
Stored (moist condition)	" " 48 "	5	"	10	0.4	9	1?

supernatant fluid of the tumor cultures in Hartley's broth was used, and in one of the experiments in which this fluid was filtered, the observation period was 34 days; in the remainder it was 2 months. In a few instances, the testicle or the skin

of the inoculation area was removed during the experiment in order to obtain additional evidence of the presence or absence of tumor growth from gross inspection or from the histological picture.

RESULTS.

These experiments which are summarized in Tables I to IV turned out entirely negative. No growth was obtained from any of the 25 injections (15 rabbits) of filtrates of tumor emulsions nor from the 15 inoculations (10 rabbits) of desiccated tumor. In like manner, no growth resulted from the inoculation of filtered or centrifuged Hartley's broth which had been in contact with tumor tissue in the ice box for 48 hours. There were 20 testicular and 10 intracutaneous injections carried out with these materials (15 rabbits). Furthermore, the tumor tissue which had been "cultured" in Hartley's broth failed to grow when injected into the testicles of 5 rabbits. On the other hand, the same tumor used in a cultivation experiment was still capable of active growth after being kept in the ice box for 48 hours under moist conditions. Primary tumors developed from 9 of the 10 inoculations made with this material.

These results therefore contrast sharply with those obtained in the control series of rabbits in which the material used for inoculation was not subjected to any manipulation other than emulsification. Primary growths were obtained in every instance from the inoculation of the same tumors which had been used for filtration, desiccation or cultivation in Hartley's broth.

DISCUSSION.

The results of the present experiments confirm the earlier observations in that it has not been possible to propagate this malignant neoplasm of the rabbit with Berkefeld filtrates of the tumor or with desiccated tumor tissue. Furthermore, no success attended the attempts to demonstrate an agent capable of growth which could be separated from the tumor cells by diffusion into a fluid nutrient medium as is the case with a filtrable chicken tumor.*

It would appear therefore that as far as this neoplasm is concerned,

* Unpublished experiments of Jas. B. Murphy.

it is reasonably certain that living cells are essential for its propagation. This deduction is supported by the results of the experiment in which active growths were obtained with tissue which had been kept in a moist condition in the ice box for 48 hours as contrasted with the failure to obtain growth from the inoculation of the same tissue which had been kept in Hartley's broth in the ice box for the same time. The probable explanation of this failure is the rapid autolysis of cells which occurs under the latter conditions.

It is significant in this connection to recall that the cells of this tumor resist supposedly deleterious influences to a remarkable degree. Repeated freezing and thawing, for instance, destroys most cells, judging from dark-field examination, but a few apparently intact cells may be recognized and intratesticular inoculation of tumor tissue subjected to these procedures is followed by tumor growth (1).

The first filtration and desiccation experiments were carried out with the 4th, 10th and 12th generations of tumor transplants, while growths of the 56th, 65th and 68th generations were used in the work now reported. Whatever changes have occurred in the growth capacity of the tumor cells incident to long continued transplantation there has evidently been no alteration in a hypothetical cell-free agent by which this agent would be more readily demonstrable with the procedures employed.

It is evident from the present experiments as well as from those previously reported, that there is an essential biological difference between this neoplasm of the rabbit and certain tumors of fowls which can be propagated with tissue filtrates or desiccates. This difference may possibly be a matter of animal species since the satisfactory demonstration of the filtrability of a mammalian tumor has yet to be made. It is not unlikely that the biological differences between such species as birds and mammals may extend to the occurrence of tumor agents distinct from tumor cells. On the other hand, the significant factor may be the type of cell involved since the fowl tumors are classified as sarcomata, while the rabbit neoplasm is considered to be of epithelial origin.

CONCLUSIONS.

It has not been possible to propagate a malignant neoplasm of the rabbit with cell-free filtrates, or desiccated tumor tissue or by the use of fluid media kept in contact with tumor tissue. These findings confirm the results of previous experiments carried out with early generations of the tumor.

The existence of an agent distinct from the tumor cell which could initiate growth has not been demonstrated.

The experiments bring out an essential biological difference between this mammalian neoplasm which is considered to be of epithelial origin and certain filtrable tumors of fowls which have been classified as sarcomata.

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[Reprinted from The Journal of Experimental Medicine, August 1, 1927, Vol. xlvi, No. 2, pp. 315–341.]

LOCAL PROGRESSION WITH SPONTANEOUS REGRESSION OF TUBERCULOSIS IN THE BONE MARROW OF RABBITS, CORRELATED WITH A TRANSITORY ANEMIA AND LEUCOPENIA AFTER INTRAVENOUS INOCULATION.

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Plates 8 to 10.

(Received for publication, May 13, 1927.)

In following the blood of rabbits after an intravenous injection of 1 or 2 mg. of bovine tubercle bacilli, we have found that there develops a characteristic anemia combined with a fall of those white cells that normally arise in the bone marrow. In previous studies, Sabin, Doan and Cunningham (1) presented evidence indicating that the epithelioid cell and its derivative, the giant cell of the Langhans type, come from the monocyte of the tissues. Cunningham, Sabin, Sugiyama and Kindwall (2) then showed that the extension of a tubercular process in the tissues is reflected in the peripheral blood, which they interpreted to mean that from an advancing tubercular area with its massive increase in normal and abnormal monocytes there is a flooding of these cells into the blood stream. Moreover they demonstrated that the healing process involving the increase of lymphocytes around tubercular tissues is also reflected in the blood so that the ratio of monocytes to lymphocytes in the blood stream may be used as an index of the state of a tubercular process in the tissues.

About 2 weeks after an intravenous injection of bovine tubercle bacilli in doses of 1 mg., the percentage and the actual numbers of monocytes in the peripheral blood of rabbits rise. At the same time the monocytes show qualitative changes which involve some lessening of motility and the production of all the intermediate morphological stages between the normal monocyte and the epithelioid cell. The cytoplasmic changes consist in an increase in the number and a de-

crease in the size of bodies, probably vacuoles, that stain characteristically in neutral red in the living cell with a gradual accentuation of the rosette about the centrosphere. In a series of 53 rabbits, Cunningham, Sabin, *et al.*, found that the percentage of monocytes in rabbits infected with bovine tuberculosis rose from an average of 8 per cent before infection to an average of 14 and a maximum of 52 per cent after infection. Their corresponding total numbers were from 943 monocytes per c.mm. before infection to an average of 1455 and a maximum of 6348 after infection. There were no qualitative changes in the lymphocytes but their numbers were increased in rabbits with high resistance to tuberculosis. The normal ratio of monocytes to lymphocytes in the rabbit is 1 to 2.97, in round numbers 1 to 3; rabbits with high resistance developed a ratio of 1 to 3.56 while the animals with low resistance showed a ratio of 1 to 0.79.

The present series of 80 rabbits were infected with 1 or 2 mg. of bovine tubercle bacilli, injected intravenously. We have used the same strain of organisms as in the previous studies, namely Strain B_1, which was isolated at Saranac but which we obtained from the Dows Laboratory of Tuberculosis, John Hopkins Hospital. Bovine Strain 214 E, secured from Dr. Paul A. Lewis, of the Department of Animal Pathology of The Rockefeller Institute, has given the same findings when used in comparable dosage in a few control animals. Beside studying the white cells in this group we have as a routine counted the red cells and taken the hemoglobin, making the studies on the average of every other day. For the counts of the red cells we have used pipettes and the Levy-Hausser counting chamber calibrated by the Bureau of Standards. The hemoglobin readings were made in a Duboscq colorimeter with the Newcomer standard about 2 weeks after the corresponding counts and by a different individual without knowledge of the totals of the red cells.

These studies have confirmed previous results (2) in demonstrating that in general the progress of the experimental tuberculosis in the tissues can be followed by the ratio of monocytes to lymphocytes in the blood. In studying animals which pass through the acute into the subacute and chronic phases of tuberculosis, it has been possible to follow further the extent of the pulmonary process, and the lesions in other organs, at various stages, and to correlate the changes with the

monocyte-lymphocyte ratio in the blood. In addition, we have found in this study that the curves of the platelets, the red cells, the hemoglobin and the granulocytic leucocytes can in turn be used as a general index of the progress of tuberculosis in the bone marrow.

In the analysis of this series, the animals may be considered in two sections: the first consists of the 12 rabbits studied for the early reaction at 24, 48, 72, 96 hours, and thereafter at intervals of 48 hours up to 18 days; the remaining 68 animals of the series fall naturally into three groups according to the phase of the anemia and leucopenia at the time of death. The progress and extent of the tuberculosis in the early reaction in organs other than the bone marrow are included in a following article (3). While the right femur was chosen for routine examination, these findings were determined to be representative of all the marrow by selected surveys covering both humeri, both femurs and the ribs.

Early Reaction.

During the first 6 days following the intravenous injection of tubercle bacilli, supravital studies of the bone marrow revealed no changes in the normal picture, except an increase, first noted at 48 hours, in the number (2 to 3 per oil immersion field) and activity of the clasmatocytes (3). Fat cells, megacaryocytes, myelocytes, erythroblasts and normoblasts were present in the usual percentage and distribution (11). Only the occasional mature monocyte, to be accounted for by the number to be found in the circulating blood, was seen. On the 6th day, however, there were found (R 217) for the first time, a few monocytes; and on the 8th day the supravital studies (R 218) showed two very definite and striking changes, undoubtedly correlated: (1) changes in the fat cells, and (2) the appearance of many young monocytes.

In this marrow of the 8th day were found, in the supravital studies, later confirmed in sections, a few clumps of fat cells showing the breaking up of the usually large, homogeneous fat globule into smaller droplets, with the beginning of the shrinking of individual cells. The demobilization of fat is obviously the principal mechanism of making room for new elements in bone marrow, as may be seen in the conversion from a fatty to a red marrow under many pathological condi-

ditions. That the fat is in an extremely labile form was demonstrated by one of us (4) in studies in the experimental hypoplasia of avian bone marrow. It was shown that in the pigeon the bone marrow of the radius may be reduced to a completely hypoplastic or fatty state during a fasting period in which there is a loss of from 100 to 150 gm. in body weight. A biopsy at this stage was followed by the giving of abundant food to the bird, with succeeding operative removals of marrow at 24 hour intervals for comparison. In 24 hours there were marked changes in the fat consisting in a regression of the fat cells toward their embryonal state. It is well known that the developing fat cell has the fat in small droplets, the nucleus being centrally placed, the ultimate resting cell showing a flattened peripheral nucleus with one, large, homogeneous fat globule within the stretched cell membrane. The regressing fat cells of the pigeon's marrow showed first a breaking up of the single, large, fat sphere into many smaller droplets. This was followed by the shrinking of the individual fat cells always toward the blood vessels to which they appeared to be anchored, and into which the fat seemed to be passing for transport from the marrow. In 48 hours all the fat had been removed from the pigeon's radius coincident with the restoration of the marrow to a rapidly regenerating hemopoiesis. It was the beginning of such a process of fat demobilization which was evident in the marrow of rabbits on the 8th day after a tubercular infection.

At the same time, the supravital studies showed marked evidence of the development of young monocytes, many of them monoblasts, with rosettes a single granule deep. No epithelioid cells were found. The normal bone marrow does not contain monocytes younger than, nor in numbers exceeding those of the circulating blood, but in this instance as many as 20 per oil immersion field were counted repeatedly. This observation with the living cells was confirmed in sections. In the fixed sections stained with hematoxylin and eosin, it would not have been possible to discriminate the individual monoblasts from myeloblasts with such assurance without the aid of the preliminary supravital studies. Nevertheless there were signs other than staining reaction in the sections themselves which indicated the interpretation to be correct. In normal bone marrow the number of myeloblasts is exceedingly limited; most of the myeloid elements are in the stage of

the late myelocyte, with a full quota of granules, which have been designated Type C (5, 6). Moreover, in myeloid stimulation or in experimental depletion of the bone marrow, myeloblasts do not appear until the myelocytes, Type C, have been reduced and the marrow thrown back to the level of the earlier Types A and B; then such a marrow, if active regeneration is to occur, shows myeloblasts and early myelocytes in mitosis and in increased numbers. In contrast, this marrow of the rabbit 8 days after infection showed only the usual percentage of late myelocytes; thus the myeloid hemopoietic elements were as yet entirely unaffected and the peripheral blood at this stage indicated no change in those blood cells coming from the marrow. Yet in the sections there were large numbers of deeply basophilic, immature forms, with centrospheres easily seen as clear areas in the dense blue cytoplasm. These were the monoblasts and young monocytes which had been seen in the vital preparations. There was no indication that they arose elsewhere than locally in the bone marrow; at this stage there was no rise in monocytes in the blood stream to indicate their transportation through the blood into the marrow. We think that they arose *in situ* from the primitive undifferentiated mesenchymal stem cell which may give rise to any of the three strains of the white blood cells, the leucocyte, lymphocyte or monocyte (6); normally this stem cell, this undifferentiated mesenchyme or so called reticular cell, gives rise only to leucocytes in bone marrow, but, under the stimulus of a tubercular infection, and after the usual very definite latent period (7), the same stem cell may give rise to monocytes, preliminary to the development of the epithelioid cell typical of the specific pathology of the disease.

Chart 1 records the data gathered from the peripheral blood in counts taken before infection and just before autopsy in the 5 animals examined from the 10th to the 18th days of the disease. On the 10th day (R 227) after infection there was a definite decrease in the fat content of the marrow with more evidence of the nature of the demobilization in the number of cells showing the breaking up of the fat into many fine droplets. In addition to increased numbers of young monocytes, some with two nuclei indicating rapid multiplication, there were numerous mature monocytes and the beginning appearance of scattered typical epithelioid cells with a full quota of mitochondria. There was a definite depletion of the late Myelocytes C; Myelocytes B were obvious, but there were no increases in Myelocytes A or in myeloblasts The red cell series showed a pre-

dominance of erythroblasts, with the appearance of megaloblasts and early erythroblasts. The clasmatocytes remained increased in number with their phagocytic activity stimulated above the normal for this tissue. The megacaryocytes, while not reduced in apparent number, showed here and there degenerating nuclei and cytoplasm.

Supravital studies on the 12th day (R 228) showed markedly decreased fat, many fields having none or only one greatly shrunken fat cell. Every field contained epithelioid cells, either singly or in small clumps; many cells had two nuclei. The myelocytes were thrown back to the B type, only a few C myelocytes being present. The neutrophilic leucocytes in the peripheral blood had fallen

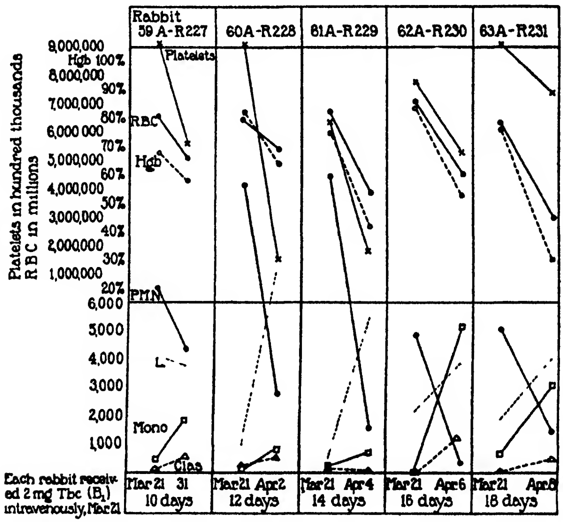

CHART 1.

from an original count of 10,000 per c.mm. to 2800 (Chart 1). The red cells while showing a larger percentage of early erythroblasts than normal still contained normoblasts in appreciable numbers. The clasmatocytes showed engorgement with whole red blood cells. Many of the megacaryocytes appeared shrunken and dead with very little cytoplasm surrounding the nuclei. The platelets had fallen from an original count of 910,000 per c.mm. to 150,000 (Chart 1).

Rabbit 61 A (R 229) (14 days) again showed a bone marrow with the fat absent from many fields in supravital surveys, the presence of fine droplets indicating the continued transportation from the marrow of the final fat deposits. Numerous typical epithelioid cells, many young forms, singly and in clumps, were in every field of every preparation. A marked reduction in C myelocytes, with many

B types, but with the predominating blood cell the early erythroblast, indicated the gradual encroachment of the new growth on the blood-forming tissues. The red cells had fallen from an original level of 6,700,000 per c.mm. to 3,900,000, hemoglobin from 75 per cent to 42 per cent, and the leucocytes from 10,500 to 1580 (Chart 1). There were many clasmatocytes, actively phagocytic, with whole, nucleated and non-nucleated red blood cells and their fragments engulfed. Only two megacaryocytes, one without cytoplasm, were seen in a survey of many fields. The platelets had fallen from an original level of 640,000 per c.mm. to 180,000.

In Rabbit 62 A (R 230) (16 days) supravital studies of the bone marrow revealed practically no fat. There were great numbers of monocytes, typical epithelioid cells and, for the first time, true giant cells of the Langhans type. There was a minimum of myeloid cells at any stage of maturity; the red cell series, largely erythroblastic with a few normoblasts, greatly predominated. The leucocytes had fallen from an original absolute number of 4800 per c.mm. in the peripheral blood to 372, while the monocytes had risen from 0 to 5084 (Chart 1). The clasmatocytes were still stimulated, and the megacaryocytes, though decreased in absolute numbers from the normal, were increasing over the percentage found at the immediately preceding stages.

At 18 days after infection (R 231) the only fat to be found in the bone marrow was an occasional fat globule the size of a red cell There were many typical epithelioid cells, showing no tendency toward phagocytosis, in sharp morphological and functional distinction to clasmatocytes loaded with phagocytosed white and red blood cells. The monocytes rose from 650 per c mm. to 3070 in the peripheral blood, and the clasmatocytes from 0 to 465 (Chart 1). There was striking limitation of the myeloid elements, baso-, pseudo- and eosinophilic myelocytes being all proportionately depressed The red cells showed all stages of erythroblastic maturation, with very few megaloblasts and normoblasts. The red count had fallen from 6,350,000 per c mm. to 3,030,000 and the hemoglobin from 76 per cent to 30 per cent, the leucocytes from 5000 to 1400. There was a striking excess of megacaryocytes in this marrow in contrast to those marrows of the period from 10 to 14 days There were many, often nests of 5 per field, small, obviously young cells, with from one to three nuclei; and then, frequently, there would be found large, single, multinucleated cells covering half an oil immersion field The platelets in the first count before infection in this animal had been 910,000 per c.mm. and in the final count they were 740,000. In the supravital preparation of the peripheral blood the platelets varied greatly in individual size, which variation was quite obvious in the counting chamber also. And this has been confirmed repeatedly in other animals in which the platelets have been followed (see for example Chart 9) During the period of low platelets in the peripheral blood there was a distinctly increased coagulation time for the blood. It will be readily seen from Charts 1 and 9, representative of 14 animals, that the platelets show a sharp decrease in the peripheral blood earlier than the

red or white cells, that the period of depression has a briefer duration (from the 9th to the 14th day after infection approximately) and that the return to the limits of normal comes often while the greatest depression of the other blood elements is being experienced. Coincident with these peripheral manifestations there may be correlated changes in the qualitative and quantitative characteristics of the megacaryocytes in the bone marrow. This evidence might be used in further presumptive corroboration of the direct relationship between the platelet of the blood and the megacaryocyte of the bone marrow, and, conversely, to question further the origin of platelets from the granulocytic and erythrocytic series.

The demonstration of the first appearance of young monocytes in the marrow on the 6th to 8th day after inoculation with massive intravenous doses of bacilli, no epithelioid cells, no tubercles yet having appeared, correlated with the constant development of marked tuberculosis of the marrow following close upon their appearance, is an added confirmation of the origin of the epithelioid cell from and through the monocyte; clasmatocytes are present in increased numbers from 48 hours on. Since the development of an extensive tuberculosis of the marrow in from 12 to 21 days after infection has been a constant finding with two strains of bovine tubercle bacilli (B_1 and 214 E) (see below), it is believed that the increase in young monocytes marks the onset of the local tubercular process. The fat is gradually depleted as the encroaching tubercular tissue advances and the anemia and leucopenia progress until the beginning spontaneous regression of the foreign tissue makes room within the rigid, bony confines of the hemopoietic organs for adequate blood formation.

The Later Reactions.

In the remaining 68 rabbits of this series, we have used the preliminary studies of the blood in each animal before infection with tubercle bacilli as the normal base line. The extent of the anemia and fall in neutrophilic leucocytes after infection are indicated in Table I. In this group, the onset of the fall of the red cells and hemoglobin has come on the average 11 days after the injection of tubercle bacilli. This average includes only 62 of the 68 rabbits, because to 6 of the animals had been given a hemolytic serum before the injection of the bacilli, thus introducing two factors in the production of the

anemia. In all 6 there was a further fall of the red cells after the injection of the bacilli but the time of onset was not so striking.

In some instances the fall of the neutrophilic leucocytes (pseudo-eosinophilic in the rabbit) began on the same day as the fall in the

TABLE I.

Blood of normal rabbits. Averages from 68 animals		Average of greatest anemia and fall of P M N in the same 68 animals after infection	
R.B.C.	5,425,697	R.B.C.	2,741,764
Hgb.	62 per cent (Newcomer)	Hgb.	35 per cent
P.M.N.	3833	P.M N.	940
	44 per cent		17 per cent

TABLE II.

Age of Cultures of Tubercle Bacilli, Strain B_1, When Used for Inoculation, with Average Period before Onset of Anemia.

Age of culture	Average day of onset of anemia
days	*day*
9	12th
13	9th
14	10th
18	11th
19	14th (All 12 animals)
28	13th
29	11th
32	10th
34	13th
38	11th
56	No anemia produced in 6 animals within 30 days

red cells, but it occasionally preceded the latter, so that the average initiation of the fall in white cells was on the 9th day. The onset of the appearance of this effect on the bone marrow has been quite constant with reference to the subculture used; for example in one instance 12 animals injected from a given subculture all showed the onset of the anemia on the same day; in every group of animals in-

fected from a given subculture 2 or more have developed the anemia simultaneously (Chart 2). There has, however, been no constant relation between the onset of the anemia and the period of incubation of the subculture (Table II); in each instance the culture chosen for inoculation has shown an excellent growth of bacilli and the effective range of incubation has been from 9 to 38 days. With a culture of 9 days the average onset of the anemia was on the 12th day, while with one of 38 days the average onset was 11 days after infection; with one old culture of 56 days there was no manifestation of the development

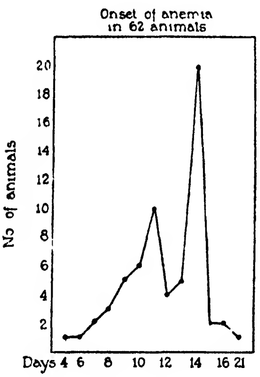

CHART 2.

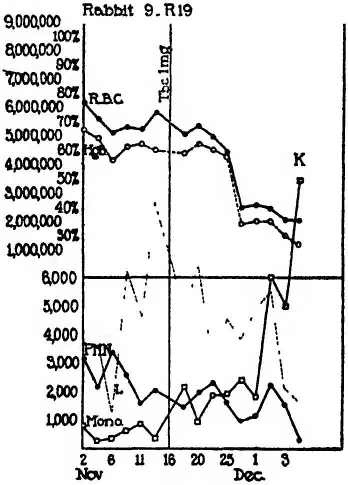

CHART 3.

of an acute phase of the tuberculosis after 30 days in the 6 rabbits inoculated (Chart 5). We have realized that to get the exact time of the onset of the anemia and leucopenia it would be necessary to have counts of the blood every day which was not done in the original series, but the general period of depression has been remarkably constant. In 2 animals of a new series counted every day the onset of the anemia was on the 9th day in both instances and the onset of the leucopenia on the 5th day.

The lowest red count in the series was 1,410,000 cells and the lowest hemoglobin was 21 per cent. There were several instances of an

extreme fall in neutrophilic leucocytes to percentages under 10 and to total numbers ranging from 100 to 200 cells per c.mm. There was one count of 100 white cells with no neutrophilic leucocytes present. The other types of granulocytes, the eosinophilic and basophilic leucocytes, have also shown changes. The eosinophilic leucocytes normally occur in small numbers in the rabbit, ranging from 1 to 2 per cent, and they disappear entirely from the peripheral blood during the leucopenic phase of the disease; the basophilic cells, normally ranging from 8 to 10 per cent, do not decrease so markedly during the leucopenic phase but, as will be shown, they are subsequently increased during the compensatory leucocytosis.

In the analysis of this latter series the animals fall naturally into three groups, according to the phase of the anemia and leucopenia at the time of death. Most of the animals died spontaneously of the disease, but a few were killed when it was estimated that they might not live through the night and supravital studies of the fresh tissues were desired. The first group, 30 animals, showed falling red cells and granulocytes at the time of death, and all died acutely during the 1st month after infection. Of this group 22 died and 8 were killed. The average length of life was 22 days, the extremes being 15 and 34 days. Only 2 of the animals lived longer than 30 days, one 31 days, the other 34 days, and neither of the 2 had a blood count for 4 days preceding death, so that the red cells may have begun to rise in them before death.

The second group consists of 17 animals which showed the beginning of recovery of the bone marrow as reflected by the onset of a rise in red cells, hemoglobin and granulocytes. Of these animals 14 died and 3 were killed. The fatalities came approximately during the 2nd month, with an average length of life of 35 days and a range of from 18 to 60 days after infection; there were 3 animals with a duration of life under 30 days; 1 was killed at 18 days, 1 died at 19 and 1 at 20 days. This group shows the remarkable fact of animals dying with an advancing pulmonary lesion while the bone marrow was recovering from tuberculosis.

The third group consists of 21 animals, in all of which the peripheral blood indicated a restoration of the bone marrow, in that the red cells, the hemoglobin and the granulocytes had either returned to or

exceeded the original normal level. The average length of life in this group was 150+ days, the range of life being from 59 to 247 days, not including 1 rabbit which was infected March 22, 1926, and is still alive after 1 year. Of these animals 14 died, 5 were killed when it was estimated that they might not have lived through the night, 1 was killed while still in good condition and 1 is still living.

On Chart 3 (R 19) is shown the record of the peripheral blood of 1 of the animals representative of the first group. The red cells and hemoglobin are shown in the upper section of the chart, the neutrophilic leucocytes (pseudoeosinophilic in the rabbit), the lymphocytes and monocytes in the lower section. Before infection the white cells ranged from 5300 on the 6th of November to 11,500 on the 13th, the increase being due to lymphocytes, not an unusual differential curve in certain normal rabbits; the monocytes were consistently below 1000. As will be seen on the chart, there was a rise in monocytes, which were entirely normal, qualitatively, on the 2nd day after infection. We have found this transitory rise in, usually normal, monocytes on the 2nd or 3rd day after infection in many of the animals of this series. This rise may be due to a division of the monocytes already in the blood stream or to the entry of new cells into the vessels from the tissues, but the survey of the general body tissues during this period (3) reveals primarily a clasmatocyte reaction, so that it would appear more probable as a reaction closely related to the blood itself. From the 1st of December there was a phenomenal rise in monocytes, a large proportion of which were abnormal with lessened motility and with markedly increased bodies staining in neutral red. There were also some typical epithelioid cells. From the 1st to the 4th of December, there was a marked rise in desquamated endothelial cells, clasmatocytes, ranging from 4 to 17 per cent of the white cells in the blood. Two of the endothelial cells from this animal have been illustrated in a preceding paper (9).[1] On December 4th, just before the animal was killed there were several sheets of endothelial cells, one of which contained 19 cells, seen in the peripheral blood, and similar sheets were seen in supravital preparations of the spleen immediately afterward. With the extreme rise in monocytes during the last 3 days, there was a fall in lymphocytes, the final ratio of monocytes to lymphocytes being 75 5 to 14 in percentage and 9475 to 1757 in numbers.

The lines of monocytes and lymphocytes on the chart show the progress of the tuberculosis in the general tissues, while the three lines of red cells, hemoglobin and neutrophilic leucocytes indicate the progress of the lesion in the bone marrow. The abrupt fall in the curve of the red cells came between the counts recorded for the 25th and the 30th, making the drop of the red cells, hemoglobin and the leucocytes come between the 9th and 14th days after infection. In the animals on which a count has been made every day for a comparable period

[1] Sabin and Doan (9), Figs. 5 and 11.

the fall has been a gradual one. The curve of the hemoglobin acts as a check on the red cell counts, the close paralleling of the two records indicating the relative accuracy of our present technical methods for ascertaining comparative values at least.

At autopsy the lungs of this animal showed diffuse generalized tuberculosis of so marked a grade that distinct tubercles were not seen in the gross; microscopic sections showed an extreme grade of the disease with only small areas with patent air sacs; there was very little caseation. The spleen was markedly enlarged, weighing 17 gm., the normal average weight being 0.9 gm. according to Rous and Robertson (10); it showed an extreme dilatation of the sinuses and extensive tuberculosis. The free cells obtained by gentle scraping of a freshly cut surface showed some epithelioid cells, large numbers of clasmatocytes with ingested red cells and many sheets of endothelial cells. On section the spleen showed extreme tuberculosis and markedly dilated sinuses. Our impression is that the marked acute splenic tumor present in every animal of the series that died during the 1st month was due to the dilatation of the sinuses The lymph glands likewise showed marked tuberculosis with caseation; also dilatation of the lymphatic sinuses. Both glands of the hilum of the lung and the mesenteric lymph nodes were studied. There were a few small tubercles in the liver; the adrenals were negative except for an occasional single epithelioid cell.

The bone marrow of this animal (R 19) is shown in Fig. 1. In the gross the marrow was dark red, elastic, not gelatinous. The striking points in the section are the complete elimination of the fat cells, the extensive tuberculosis and the reduction of the marrow to an early erythroblastic level The supravital preparations showed no fat, but there were epithelioid cells as evidence of the tuberculosis and early erythroblasts with an occasional megaloblast In contrast to normal bone marrow, there was a great reduction in myelocytes. A few myelocytes are seen in a gray tone across the artery in the center of the section. The edge of the marrow, which is usually marked by a prominent zone of mature myelocytes, will be seen in this section to be markedly depleted. The section shows two types of areas, the paler zones which are tubercular tissue, the darker zones which are masses of early erythroblasts There are some megacaryocytes, but our impression is that they are reduced in number. Such a bone marrow corresponds with the peripheral blood on the last day of Chart 3; the leucocytes were reduced to 2.5 per cent with 313 as the total number; the red cells were 2,040,000 and the hemoglobin 27 per cent. In the normal marrow there are many times more myelocytes of Type C (6) than nucleated red cells; in this marrow the supravital studies and the section show vastly more erythroblasts than myelocytes but the erythroblasts were for the most part too young to have been ready for the peripheral blood. Thus this marrow had been depleted of its fat to make room for the tubercular tissue and at the same time the marrow had been depleted of most of its store of myelocytes and normoblasts: it had been thrown back to the level of the early erythroblasts with no sign whatever of a

stimulation of them into the late erythroblasts and normoblasts. Moreover, there was a tendency toward the formation of myeloblasts to indicate a regeneration of the white series. From such a marrow the anemia and the low level of granulocytes in the peripheral blood are readily understood. This marrow stained for tubercle bacilli showed often as many as 10 or 12 acid-fast organisms in the occasional epithelioid cell.

On Chart 4 is shown the blood of a second rabbit (R 18) of the first group. It shows the same points except that the terminal rise in monocytes was not quite so marked though the animal died with the monocytes exceeding the lymphocytes in the peripheral blood. These two charts are entirely representative of the complete group of 30 animals that died in the acute phase of the disease.

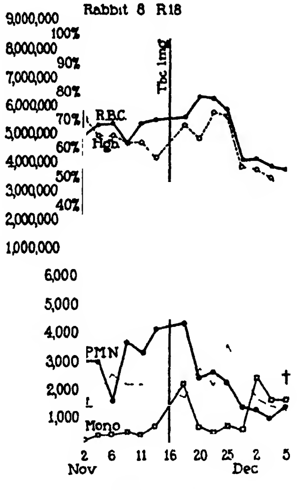

CHART 4.

In every instance there has been extreme or miliary tuberculosis of the lungs; acute splenic tumor with marked tuberculosis; involvement of the lymph glands and extensive tuberculosis of the bone marrow. All the charts of the peripheral blood of this group are practically identical: they show a fall in red cells, hemoglobin and granulocytes which has been correlated at autopsy with the demonstration of a marked tuberculosis of the bone marrow; as a sign of the general tuberculosis, there has usually been the transitory rise in monocytes on the 2nd day, with a marked rise in abnormal monocytes in the 3rd week and a corresponding fall in lymphocytes.

The second group of rabbits, the 17 that survived the first acute hemopoietic depression from the infection, yet died for the most part

in the 2nd month, all showed the beginning of a recovery of the bone marrow.

On Chart 6 (R 80) is the record of the peripheral blood of an animal showing the beginning change from the reaction which occurred in the first group Before infection this animal received two doses of a hemolytic serum which caused some fall in both red cells and hemoglobin. The blood was counted twice on the day of the injection of the bacilli. At 9 00 a m. the total white count was 6150; the bacilli were given at 9 30, by 1 30 the white cells numbered 16,500, and the chart shows that the rise was due to a transient outpouring of the neutrophilic leucocytes. The onset of the anemia and the fall in leucocytes secondary to the

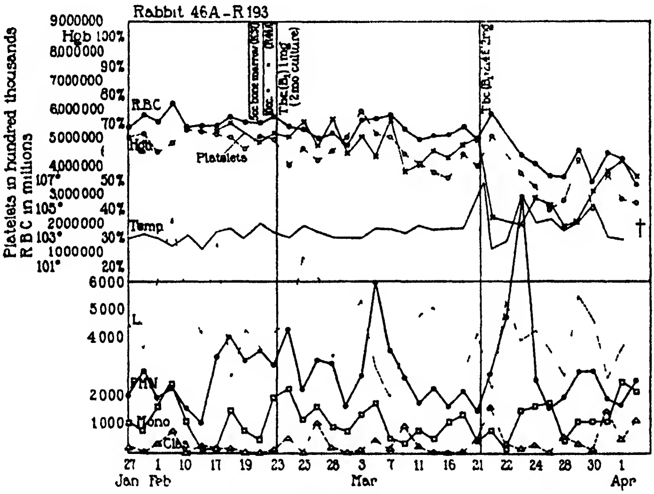

CHART 5.

tuberculosis began on the 9th day. The interesting point in the chart is that there was a slight rise in the red cells, more marked in the hemoglobin just before the death of the animal. A rise in hemoglobin accompanied or followed by a rise in the red cells is constant for the entire group. The terminal rise in leucocytes shown on this chart is unusual for animals dying at this stage. At autopsy, this animal showed extensive tubercles of the lungs, some of which were caseated and surrounded by lymphocytes; this is in contrast to the extreme diffuse reaction of the first group. The spleen had marked dilatation of the sinuses, extensive tuberculosis of the pulp, with some involvement of the follicles. The marrow was most interesting (Figs. 4 and 5). With the low power, the marrow is not far different from that of Fig 1; there is the same extensive tuberculosis, the same

complete elimination of fat and the same reduction of the marrow to an early erythroblastic state. The distribution of the epithelioid cells is rather more diffuse, and this is characteristic of most of the marrows in the early stage; it indicates that there is no reaction whatever on the part of the connective tissue frame work in the marrow. The differences between this marrow and that of Fig. 1 are shown in the higher power of Fig. 5 and consist (1) in the signs of degeneration of the single epithelioid cells and (2) in the presence of normoblasts. The large pale epithelioid cells are very clear with the reduced and altered chromatin of the nuclei and the vacuolated cytoplasm In both marrows there are certain pale areas, from which the epithelioid cells seem to have disappeared entirely as if bone marrow was unfavorable soil for their persistence. There are no signs of

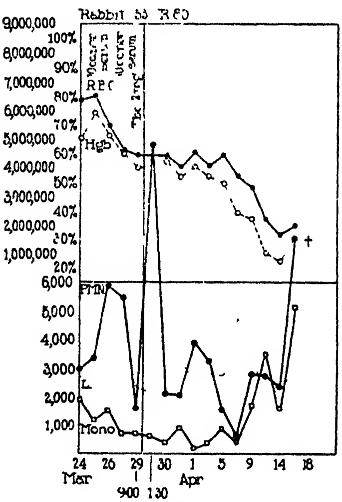

CHART 6

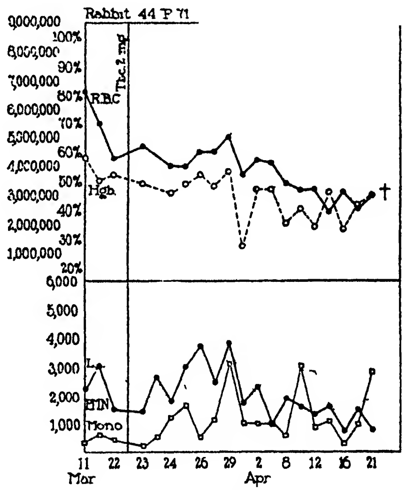

CHART 7.

any degeneration of the white cells, but in Fig. 5 there are two swollen endothelial cells, which have been shown (4, 11) to mark the onset of the regeneration of red cells, and certain lines of normoblasts are quite clear along the left border of the section. It is interesting to note that so slight a change in the red cells as is shown in the chart is readily found in the bone marrow. When this marrow was stained for tubercle bacilli the results were most interesting. Whole bacilli in epithelioid cells were rare. On the other hand the vessels were outlined by cells filled with acid-fast granules in the Ziehl-Neelsen technique. These cells occur along the large venous sinuses, the smaller veins and capillaries and even along the collapsed capillaries which Doan (4) first described as passing between individual fat cells. They make the vascular pattern almost as plain as an in-

jection. The cells with the granules are the clasmatocytes, the so called adventitial cells of Marchand, and occasionally it appears as if the endothelium itself might contain some of the granules. Evidence is presented in a following paper (3) indicating that the clasmatocyte fragments tubercle bacilli in this manner while the epithelioid cell retains them intact.

The same processes, carried a little farther, are shown on Chart 7 (R 71). The corresponding bone marrow is shown in Figs. 6 and 7. The animal lived for 31 days after inoculation and the chart shows a definite rise in hemoglobin, and the very beginning of a rise in the red cells. The lungs had an extreme tubercular pneumonia and no lymphoid reaction. The spleen showed dilatation of the sinuses and tuberculosis of the follicles In the bone marrow (Fig. 6), it is plain that there are two different types of areas· first, the large tubercular zones such as the one on the left with a few fat cells in the center; second, zones such as the center of Fig 6 and nearly all of Fig. 7 (higher power) from which the epithelioid cells have disappeared entirely, leaving the normal reticular framework of the marrow In these latter areas the clumps and lines of developing red cells with their deeply staining nuclei are obvious Beside the red cells there are small clumps of early myeloid elements, myelocytes, Types A and B, much too young to be giving rise to leucocytes for the blood stream Again the more advanced regeneration of the marrow is in the red elements as is obvious in Fig 7, but neither red cells nor white cells are sufficiently advanced to have made a marked change in the blood.

The comparison of Figs 5 and 7 brings out what happens to tubercular tissue in the bone marrow: the individual epithelioid cells degenerate as shown in Fig. 5 without any evidence of caseation; the debris is quickly cared for as formed and there are left the zones so obvious in Fig. 7 which reveal the reticular framework quite unchanged. In all the marrows, the early erythroblastic group has persisted throughout the acute phase of the disease, only a few megaloblasts having been encountered, that is to say the production of red cells has only rarely been completely thrown back to the level of the parent endothelium, a stage produced in the pigeon by underfeeding (4), and described for the human by Peabody (12). In general, in the animals of our series, the first step in regeneration (excluding the platelets) has been a rise in the hemoglobin, showing that the first compensatory mechanism is an increase in the amount of hemoglobin per cell; the next step is the increase in the number of the red cells and the last the rise in leucocytes. This relative reaction time of red cell *versus* granulocyte follows both the embryonic and the regenerative potentialities of these respective cell types as shown by Sabin (13) and Doan (4, 11).

Further stages in the recovery of the marrow are shown on Charts 8 (R 20), 9 (R 196) and 10 (R 12). On Chart 8 the rise in hemoglobin and red cells is now definite; the leucocytes have stopped falling and are perhaps just beginning to return to the blood. Chart 9 depicts a further recovery in red cells and hemoglobin, with definite recovery in the neutrophilic leucocytes while the animal

was dying of acute pulmonary tuberculosis. This graph is representative of the group of animals in which findings with reference to blood platelets and temperature were studied. In 14 animals in which the platelet count was followed there was uniformly an earlier (average 7 days) and more sudden depression of these elements in the peripheral blood than of the other bone marrow derivatives, though the period of greatest depression usually coincided with a falling red and neutrophil count. The recovery of the platelets both as observed in the peripheral blood and as correlated with the disintegration and regeneration of megacaryocytes in the bone marrow (see Chart 1) indicates a more sensitive mechanism here in its response to the general depression of the disease with a more vigorous and immediate readjustment to the new conditions. In this respect it will be

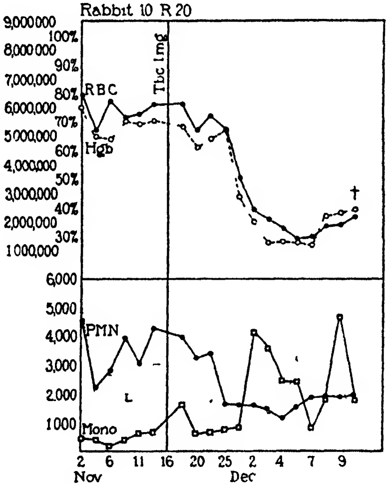

CHART 8.

noted (Chart 9) that the temperature response coincides with the sharp fall in platelets and neutrophils while the red cells are only beginning to show a more gradual decline. The elevation of temperature shows the higher range during the gradual depression of the elements from the bone marrow in the peripheral blood, a period corresponding to the rapid development and extension of the tubercular process in the other organs and tissues of the body as well. Chart 5 (R 193) indicates the lack of elevation of temperature and the uniform blood picture relative to all the bone marrow elements after a 1 mg. injection of bacilli from a culture 56 days old. A reinfection with 2 mg. of a young virulent culture of the same organism promptly initiated the usual changes in the blood with an immediate fall in platelets and a rise in temperature. The clasmatocytic

shower on the day following the reinfection (March 22) is represented by the cell reproduced in another paper (3).[2] This animal died during the acute progression of the tubercular process in the bone marrow. The other 5 animals of this experiment all died acutely within 12 hours after the second reinfection, obviously being in an allergic state even though the blood picture had remained within normal limits and the tubercular process relatively quiescent.

These changes both in the red cells and in the leucocytes are well marked on Chart 10, where the red cells are practically at the original level of between 4000 and 5000. The bone marrow corresponding to Chart 10 is shown in Fig. 2. This animal was in very poor condition, was losing weight and had very sluggish circulation, so it was killed. The lungs showed large and small tubercles sur-

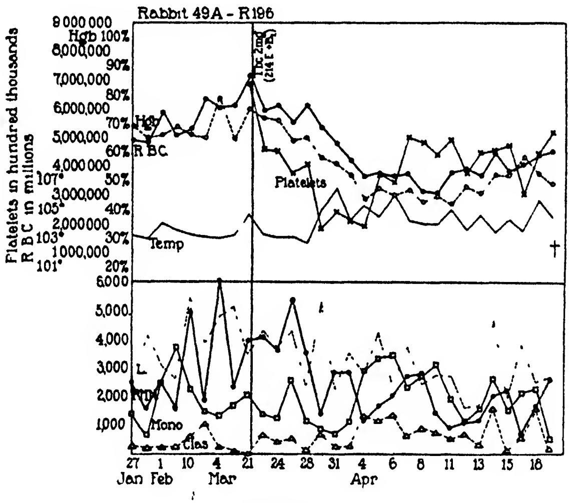

CHART 9.

rounded by lymphocytes. There was very little caseation; no pneumonia. The spleen had very few tubercles; in supravital films most of the free cells were of the clasmatocyte type with but few monocytes. The bone marrow in supravital studies showed the return of the fat; there were large areas of mature myelocytes, some zones of the primitive reticular cells and myeloblasts. Among the red cells were many normoblasts. There is an oblique line across the section to the left of which is a depleted zone from which it is probable that epithelioid cells have just disappeared. There are a few tubercles in the marrow, none showing in this photograph. To the right of the oblique line is an area in which the

[2] Sabin and Doan (3), Fig. 5.

myelocytes predominate over the clumps of nucleated red cells as in normal bone marrow; the area is, however, hyperplastic. To the left of the oblique line the marrow is still considerably depleted and there are patches of younger myelocytes, Types A and B. In both R 20 and R 12 the tuberculosis of the lung was in the form of tubercles rather than the diffuse reaction. The spleen of the animal from which Chart 8 was taken was still enlarged; the one corresponding to Chart 10 was of normal size and contained only a small amount of tubercular tissue.

The last animal of the second group to be illustrated is shown on Chart 11 (R 68), with the corresponding bone marrow in Fig. 3. In this animal it is very clear that the hemoglobin started to rise before the red cells themselves. At the time of death, which was 59 days after infection, the red cells were back to

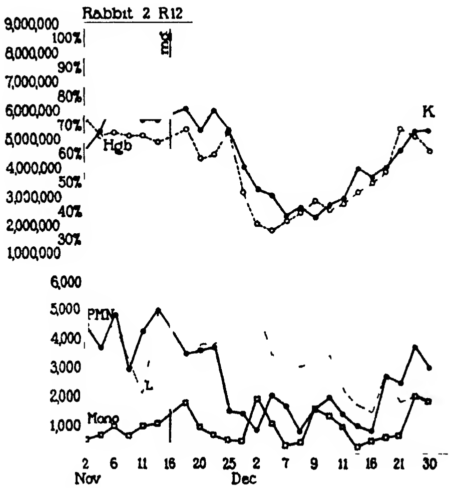

CHART 10.

the original level, while the neutrophilic leucocytes were varying around 6000 in contrast to the original base line of 3000. It will be noted that after the marked rise of monocytes in the 3rd week, they remained high and the lymphocytes dropped, indicating low resistance, and at autopsy both lungs were riddled with tubercles, with large areas of caseation and cavitation. The spleen was of normal size; there were caseous tubercles in the kidneys. On the chart has been added the line of the desquamated endothelial cells, or clasmatocytes. It will be noted that on April 12 the clasmatocytes rose together with the characteristic increase in monocytes of the 3rd week of the disease and then gradually fell to normal numbers. This period of the first great rise in monocytes after massive infection coincides with the period of the extreme diffuse lesions in the lung and

with the acute splenic tumor. At that time there is always the rise both in monocytes and clasmatocytes, together with degenerating types that cannot be analyzed and much cellular debris in the peripheral blood. This is the period of the anemia and the supravital films of the blood always show increased fragmentation of the red cells even when the preparation is looked at immediately and when the temperature of the warm box is carefully regulated so that it does not exceed 37° (14). Beside the free fragments of the red cells in the films of blood, we frequently find some of the clasmatocytes of the blood stream filled with them. Films of the lungs and spleen at this stage always contain clasmatocytes filled with fragments of red cells, the possible stimulus to their increase.

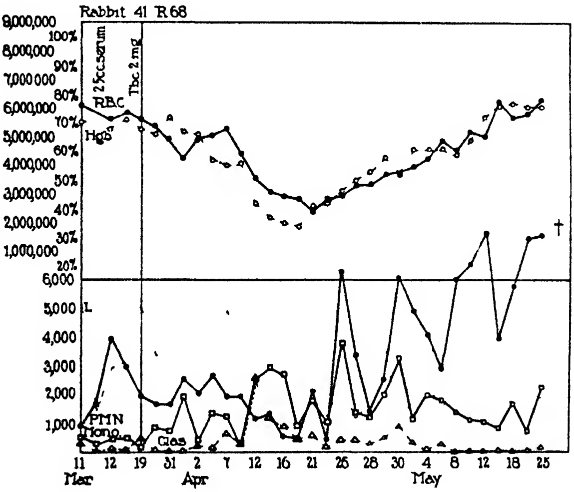

CHART 11.

The bone marrow of this animal (R 68), as will be seen in Fig. 3, is hyperplastic. In the center of the photograph is a small tubercle; the rest of the section shows the gray masses of myelocytes, many of which are still of the younger Types A and B, and the normal proportion of groups of erythroblastic cells. The interesting point in this second group of animals is the universal tendency to a spontaneous healing of the bone marrow of rabbits, irrespective of the eventual fatal progress of the disease in the animal body elsewhere.

When the rabbits survive beyond the 2nd month into the more chronic phase of the disease, the bone marrow passes through the hyperplastic state to the normal. This phase is illustrated in Chart 12 (R 11) and Fig. 8 (R 11), Fig. 9 (R 97) and Fig. 10 (R 95). On Chart 12 is shown a typical picture of the peripheral blood in a rabbit of good resistance; the animal was killed 110 days after

inoculation while still in good condition. In regard to the indications on the chart of the general tubercular infection, there was the usual sharp rise in monocytes on the 2nd day after infection; the monocytes then fell to normal until the .st of December when there was the characteristic rise of the 3rd week. From the 6th to the 16th of December, the period of greatest leucopenia, it will be noted that the monocytes were again normal and the ratio of monocytes to lymphocytes was entirely normal; this is the period of recovery from the first extreme reaction of the lungs. Then follows the record of a slowly progressing tuberculosis of the lungs in the gradually rising monocytic curve. At autopsy the left lung was largely air-containing; the right was riddled with tubercles, and in sections it appeared that the center of each tubercle was filled with leucocytes

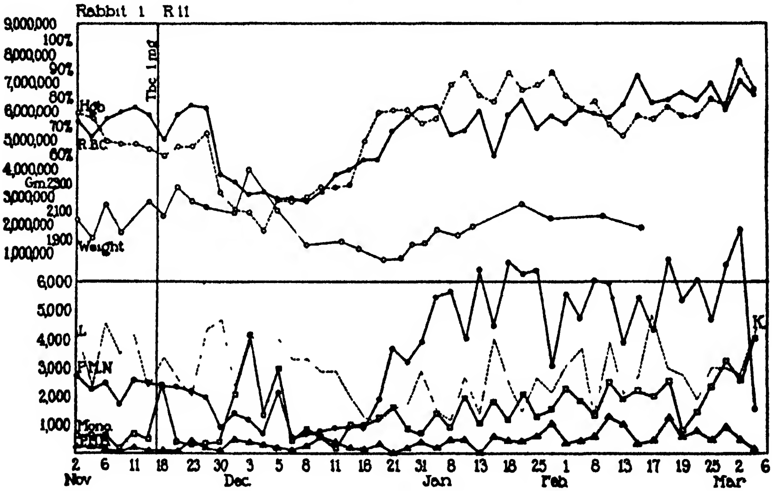

CHART 12.

instead of showing the usual caseation. The liver and spleen did not show any tubercles; there was some myeloid reaction in the spleen. The mesenteric lymph glands were loaded with clasmatocytes filled with brown pigment but no tubercles were found. In the bone marrow no tubercles were found in sections and no epithelioid cells in the fresh films.

As a record of the functional activity of the bone marrow, it will be seen on the chart that following the period of the anemia and fall of the leucocytes, the marrow recovered its hemopoietic activity and passed into a stage of hyperactivity which was still present when the animal was killed. The lowest line of the chart represents the basophilic leucocytes and shows that though they do not disappear as do the eosinophils during the period of the leucopenia, they, with

the eosinophils, increase during the general hyperplasia of the marrow. In the section of the marrow, Fig. 8, it will be noted that there is a return of the fat cells, though not yet to their normal numbers. No tubercles were found in the sections; the marrow is hyperplastic in still more marked grade than was shown in Fig. 3. There are vast gray areas of myelocytes, largely of Type C with many metamyelocytes. Among the red cells the predominating type was the late erythroblast with normoblasts. The final stage of the disease as far as the bone marrow is concerned involves the complete return to the normal structure with a normal supply of cells to the peripheral blood.

The marrow of Fig. 9 is from a rabbit that lived 135 days and of Fig. 10 from an animal that lived 153 days. In both animals the findings in September after the summer interval showed that the marrow was giving a normal output of red cells and granulocytes to the blood. The rabbit (R 97), from which the marrow of Fig. 9 was taken, had 4,970,000 red cells and a hemoglobin of 61 per cent. The white cells were 11,500, of which the neutrophils were 55 per cent, the lymphocytes 16 per cent and the monocytes 29 per cent. The reversal of ratio of monocytes and lymphocytes is the striking feature. The animal was gaining in weight. The autopsy showed restricted tuberculosis of the lungs but well marked active renal lesions. In all the sections of the bone marrow we found only one tubercle, which is shown in Fig. 9; all the rest of the marrow was entirely normal in appearance. However, in the Ziehl-Neelsen stain the clasmatocytes showed the same acid-fast fragments, though in decreased numbers, as have been described for the early stage.

The rabbit from which the marrow of Fig. 10 was taken also showed a normal output of cells from the bone marrow. The red cells were approximately 6,000,000, the hemoglobin 57 per cent; the neutrophilic leucocytes 47 to 57 per cent; the lymphocytes 9 to 15 per cent and the monocytes 25 to 30 per cent, again a striking reversal of lymphocytes and monocytes. The animal was killed on account of marked tuberculosis of the eyes. The bone marrow appeared entirely normal in the gross and supravital studies, and the sections confirmed this. In this marrow there were still a few clasmatocytes along the vessels containing acid-fast granules.

SUMMARY.

In this series of rabbits it was found that the rabbits dying during the 1st month after an injection of 1 or 2 mg. of bovine tubercle bacilli show the same conditions: extreme tuberculosis of the lungs, acute splenic tumor with tuberculosis, involvement of the lymph glands, an occasional small tubercle in the liver and extensive tuberculosis of the bone marrow. The peripheral blood has shown a sharp fall in the platelet count, an anemia and a fall in the granulocytic strain of white cells, and these changes have been correlated with the condition of the

bone marrow. There has also been a rise in monocytes and a fall in lymphocytes, to a reversal of the normal ratio.

When the rabbits have survived the first acute phase of the disease longer than 3 to 4 weeks, there have been signs in the peripheral blood of a recovery of the bone marrow; the first indication of this has been an increase in platelets, then a rise in hemoglobin followed in 1 or 2 days by a rise in red cells and later a return of the three strains of granulocytes. The bone marrow has shown a rapid spontaneous disintegration of the epithelioid cells correlated with the appearance of increased evidence of acid-fast debris in clasmatocytes, especially clear in those that lie along the vessels.

The animals that have survived into the 3rd month have all shown a hyperplastic phase of the healing marrow, both the red cells and all types of the granulocytes appearing in the peripheral blood in numbers above the normal. The epithelioid cells originally containing many bacilli all disappear from the marrow and the only sign left, possibly suggestive of the tuberculosis, is the acid-fast granules in the clasmatocytes. Finally, the marrow becomes entirely normal, giving the normal number of red cells and granulocytes to the blood. Thus, bone marrow in the rabbit has become involved in every instance with the injection of massive doses of viable bacilli. The findings at autopsy in those animals followed during the early reaction to infection confirm this directly and, since the curves of the cells in the peripheral blood of the more chronic animals were the same during the early stages of the disease as in those that died, the same conclusion seems justified from indirect inference for them. The method of healing has been a rapid disintegration of the epithelioid cells without caseation. The bone marrow heals itself entirely regardless of the progress of the disease elsewhere, so that one sees the remarkable condition of an animal recovering from the anemia and leucopenia while dying of tuberculosis elsewhere. The spleen also shows a tendency toward spontaneous healing. In the animals that have lived beyond 100 days there has been some gradual lessening of the diffuse distribution and extent of pulmonary lesions with the development of cavitation together with a marked involvement of the kidneys and lesions in the eyes.

CONCLUSIONS.

1. With massive intravenous injections (1 to 2 mg.) of bovine tubercle bacilli in rabbits there is a marked involvement of the bone marrow in the early acute phase. This reaction is initiated on the 8th to 10th days by the development of large numbers of young monocytes *in situ*.

2. From the 12th to the 20th day, approximately, there is an increasing development in bone marrow of typical tubercular tissue, epithelioid cells and giant cells of the Langhans type, many showing tubercle bacilli. This new growth eliminates the normal fat cells and encroaches upon and depresses the hemopoietic foci.

3. The bone marrow always tends toward spontaneous healing provided the animals survive the first acute reaction sufficiently long.

4. The method of healing involves a rapid disintegration of the epithelioid cells without caseation and the phagocytosis of debris by the clasmatocytes.

5. The extent and progress of the tuberculosis of the marrow are accurately reflected in the peripheral blood by a decrease of platelets, an anemia and a fall in the granulocytic leucocytes.

6. The onset of the recovery is initiated by the return of the platelets to normal, by a rise in hemoglobin, followed quickly by a rise in red cells, and by a more gradual increase in the granulocytes.

7. During the 3rd month, and after, there is a hyperplasia of the blood-forming elements in the bone marrow with a rise in the peripheral blood of the red cells, hemoglobin and the granulocytes above their original levels.

8. The bone marrow becomes entirely normal when the animal survives beyond 100 days, regardless of a steadily progressing, extensive tuberculosis elsewhere.

9. The varying length of survival in this series of rabbits under uniform environmental conditions, and infected with the same dosage of the same strain of organism, tends to emphasize the importance of the factor of individual resistance of the host in susceptibility to infectious disease.

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EXPLANATION OF PLATES.

Plate 8.

Fig. 1. R 19, hematoxylin and eosin, × about 120. Length of life 18 days. Bone marrow showing extensive, diffuse tuberculosis. Note absence of normal content of fat and myeloid depression.

Fig. 2. R 12, hematoxylin and eosin, × about 120. Length of life 44 days. Bone marrow with returning fat cells after regression of the local tubercular process, with hemopoietic hyperplasia.

Fig. 3. R 68, hematoxylin and eosin, × about 120. Length of life 59 days. Bone marrow showing one remaining tubercle, without surrounding cellular reaction; marked hyperplasia of myeloid and erythroid foci and beginning reappearance of fat cells.

Plate 9.

Fig. 4. R 80, hematoxylin and eosin, × about 120. Length of life 20 days. Bone marrow with absence of fat cells and depression of hemopoiesis by invading tubercular tissue.

Fig. 5. R 80, hematoxylin and eosin, × about 875. Detail of Fig. 4. Beginning regression of the tubercular invasion, showing partition of chromatin in disintegrating nuclei and vacuolated cytoplasm of the epithelioid cells.

Fig. 6. R 71, hematoxylin and eosin, × about 120. Length of life 31 days. Bone marrow with local regression of the epithelioid cells showing open areas of regenerating red cells and returning fat even in the midst of intact tubercular areas.

Fig. 7. R 71, hematoxylin and eosin, × about 260. Detail of open area in Fig. 6, showing intravascular limitation of developing erythroblasts, a noncellular matrix marking the former site of invasion of the epithelioid cells.

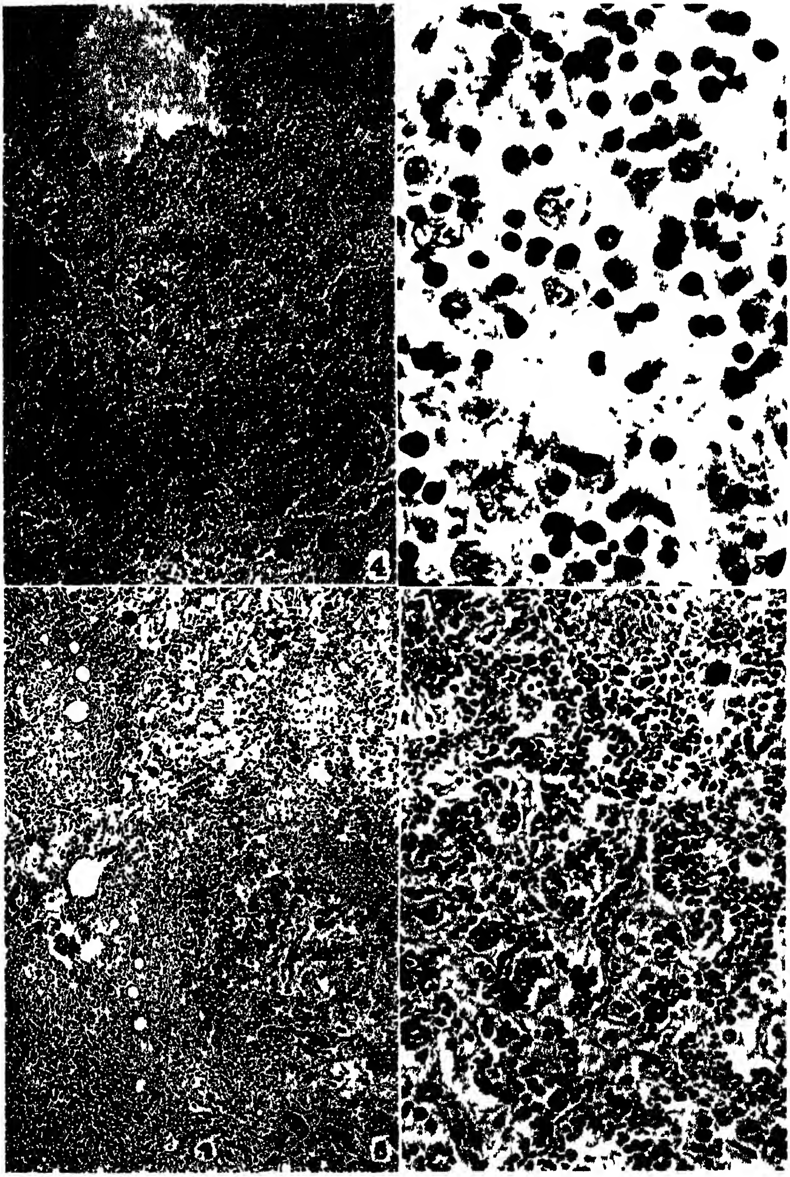
Plate 10.

Fig. 8. R 11, hematoxylin and eosin, × about 120. Length of life 110 days. Bone marrow hyperplasia of blood-forming elements, no evidence of tuberculosis remaining locally.

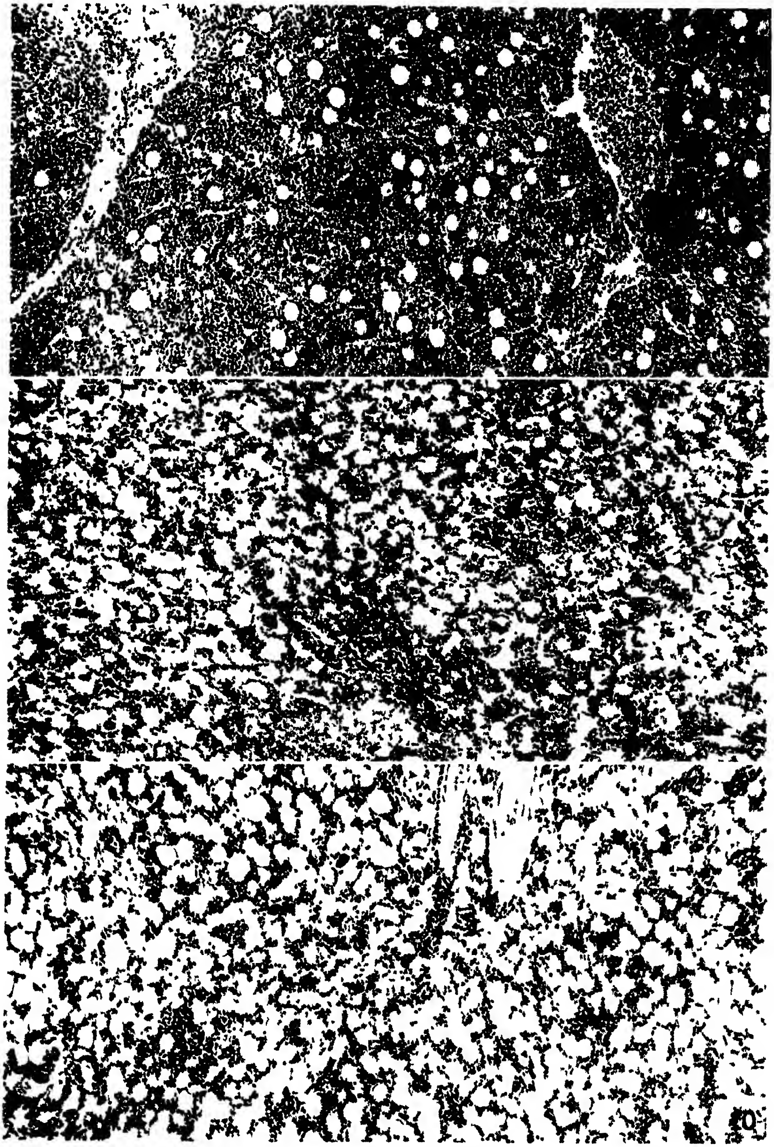
Fig. 9. R 97, hematoxylin and eosin, × about 120. Length of life 135 days. One tubercle with lymphoid focus in otherwise essentially normal bone marrow.

Fig. 10. R 95, hematoxylin and eosin, × about 120. Length of life 153 days. Return of bone marrow to normal.

(Doan and Sabin Tuberculosis)



(Doan and Sabin: Tuberculosis.)



(Doan and Sabin: Tuberculosis.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1927, Vol. xlvi, No. 3, pp. 391–407.]

LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

I. EXPERIMENTAL PNEUMOCOCCAL MENINGITIS IN RABBITS.

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PLATES 11 TO 15.

(Received for publication, May 31, 1927.)

Pneumococcal meningitis is not a common disease, and yet a certain number of cases appears each year in most larger hospitals. The disease is quite uniformly a fatal one. Whereas occasionally descriptions of recovery appear in the literature, one is frequently left with the impression that diagnosis was either incomplete or incorrect in such instances. To all intents and purposes, therefore, the disease may be considered as having a mortality of nearly 100 per cent. Consequently, any treatment, drastic as it may at first appear, which offers hope of benefit to even a small number of these unfortunate patients, may be worthy of consideration.

The problem of advancing the therapy of pneumococcal meningitis has interested this laboratory beginning with the study of Lamar (1). Lamar's work was done long before our far fuller knowledge of the pneumococci was obtained. His study was therefore somewhat incomplete from our present day point of view Lamar was impressed by the fact that the pneumococcus was bile-soluble. It occurred to him, therefore, to attempt to influence the course of experimental pneumococcal meningitis by the intrathecal administration of mixtures of immune serum, sodium oleate, and boric acid. He had already noted (2) that pneumococci treated with sodium oleate became more subject to autolysis and that they became at the same time susceptible to serum lysis. This serum lysis tended to be incomplete with normal serum, but when immune serum was employed, lysis was complete, no multiplication of organisms took place in the test-tube mixture, and an inoculated animal was protected from infection. However, the soap action was inhibited in the presence of protein, and Lamar developed a method of minimizing this protein inhibition by adding to the serum-sodium oleate an appropriate quantity of boric acid. A number of monkeys was infected

directly in the lumbar subarachnoid space with a culture isolated from a human case of pneumococcal meningitis; the organism was not typed; it had only moderate mouse virulence, but produced in the monkey a fatal meningitis, closely resembling pneumococcal meningitis in man, save that the course of the experimental disease was more rapid. Lamar found that repeated administration of mixtures of sodium oleate, immune serum, and boric acid would arrest established infections and lead to enduring recoveries.

The value of this treatment may be determined only from its employment in human cases of the disease, and apparently little benefit followed its use (Kolmer (3)). Nor in our opinion have subsequent developments in therapy, such as Kolmer (3) and Kolmer and Idzumi (4) advocate, namely, saline lavage, or combinations of saline lavage with the administration of the antibody solution of Huntoon, or with various chemotherapeutic agents given much promise of beneficial results in man. Kolmer's results will be more fully discussed in a subsequent paper.

In view of all these facts, it was decided to reinvestigate pneumococcal meningitis. The first procedure consisted in a search for a satisfactory laboratory animal. Cats proved too resistant for our purposes; whereas with large doses of Type I pneumococci injected directly into the cisterna magna it was possible to produce acute leptomeningitis, the results were irregular and it was felt that it was not justifiable to draw conclusions from so resistant an animal. Attempts to produce the disease in guinea pigs were abandoned for similar reasons. The rabbit was at first reserved because of its high susceptibility, but was, nevertheless, next employed. In our experience the rabbit presents a difficult problem so far as highly virulent Type I pneumococcal infections are concerned in that, though extremely responsive to pneumococci, the production of meningeal disease in any way resembling that found in man was not readily attained.

If a normal rabbit receives Type I pneumococci in the cisterna magna, the development of septicemia is very rapid, and death is septicemic rather than meningeal. The cellular reactional processes common to human pneumococcal meningitis, as a rule, do not occur. Such, however, appears not to be the conclusion of Idzumi (5), for the latter reports vigorous leucocytic reactions due to Type I pneumococci in the rabbit meninges. Idzumi's pneumococci were apparently of low virulence, the mouse M.L.D. for his Type I being 0.001 cc. in 24 to 30 hours. It was employed in relatively enormous dosage—dosage at least 100 times as great as we have required to produce a fatal

septicemia without much meningeal reaction in a normal rabbit. In addition, Idzumi used a 24 hour culture, undoubtedly containing many damaged cocci. More recently pneumococcal meningitis has been studied in the rabbit by Untersteiner (6). The organism used was not typed, and we are therefore unable to analyze his results in terms of our own.

EXPERIMENTAL.

In studying pneumococcal meningitis of rabbits from the point of view of therapy, one is limited by several considerations: (1) the extreme susceptibility of the animal; (2) the invariable development of massive septicemia when virulent organisms are used—a septicemia which kills before the reactional process in the meninges develops to a sufficient extent to dignify it by the term "meningitis;" (3) the difficulty in lumbar punctures in the rabbit; (4) the limited working space for repeated injections into the cisterna magna resulting in frequent trauma; and (5) the rather soft consistency of the rabbit brain, rendering pressure deformation frequent.

It became apparent early that pneumococci injected into the rabbit's cisterna reached the blood stream in enormous numbers, beginning almost immediately and resulting in massive septicemia within so short a time as 5 hours after infection. Animals died within less than 24 hours, the anatomical cause of death being apparently intense edema and congestion of the lungs. Pneumococci could be demonstrated readily in the capillaries of all the large viscera (Figs. 1 and 2) and in blood smears. These findings led us to adopt methods of preventing the septicemia or of minimizing it to such an extent as to render it a negligible factor in the death of the rabbit. Naturally, recourse was had to partial immunization of the animals either passively, or actively. Rabbits were passively immunized by the intravenous administration, either just before cisternal infection, or a few hours previously. Usually 10 cc. of Type I antipneumococcus serum (therapeutic) was used. This serum was kindly supplied us by Dr. Augustus Wadsworth, of the Laboratory of the New York State Department of Health. Its potency was such that 0.2 cc. protected mice against simultaneous inoculation of 0.2 cc. of standard culture Active immunity was established by one or two series of six intravenous

TABLE I.

No	Type	Method of immunization	Infecting dose	Site of infection	Result of blood cultures					Duration of life	Post-mortem heart blood culture	Meningeal reaction
					1–1½ hrs.	2½–3 hrs	5–5½ hrs.	24 hrs	48 hrs.			
1	Actively immune	6 injections, 1 cc. vaccine, intravenously	0.5 cc.; 1:50, 18 hr. culture	Cistern	0	0	5	0	0	17 days	0	0
2	Control	None	0.5 cc.; 1:50, 18 hr. culture	"	25	370	∞			27 hrs.	+	+
3	Actively immune	6 injections, 1 cc. vaccine, intravenously	16,000,000, 18 hr. culture	"	0	0	0	0		32 days	0	0
4	Control	None	16,000,000, 18 hr. culture	"	0	0	0	4620		45 hrs.	+	++
5	Actively immune	12 injections, 1 cc. vaccine, intravenously	111,000,000, 18 hr. culture	"	60	810	4020			22 "	+	++++
6	Control	None	111,000,000, 18 hr. culture	"	31,800	∞	∞			22 "	+	+
7	Actively immune	12 injections, 1 cc. vaccine, intravenously	55,000,000, 18 hr. culture	"	0	0	Accidental death			5½ "	+	0
8	Control	None	55,000,000, 18 hr. culture	"	0	920	∞			26 "	+	+
9	Actively immune	12 injections, 1 cc. vaccine, intravenously	81,000,000, 18 hr. culture	"	20	165	375			22 "*	+	++
10	Control	None	81,000,000, 18 hr. culture	"	530	∞	∞			22 "	+	++
11	Passively immune	5 cc. N.Y.S. serum intravenously	35,000,000, 18 hr. culture	"	20	535	900			22 "	+	++++

12	Passively immune	5 cc. N.Y.S. serum intravenously	35,000,000, 18 hr. culture	Cistern	0	750	4640	60		44 days	+	++
13	Control	None	35,000,000, 18 hr. culture	"	55	1600	42,840			22 "	+	+++
14	Passively immune	10 cc. N.Y.S. serum intravenously	26,000,000, 18 hr. culture	"	5	290	2180	60		46 "	+	+++
15	Actively immune	6 injections, 1 cc. vaccine, intravenously	26,000,000, 18 hr. culture	"	0	15	15			26 "	0	++++
16	Control	None	26,000,000, 18 hr. culture	"	2320	∞	∞			22 "	+	++
17	Passively immune	10 cc. N.Y.S. serum intravenously	13,000,000, 18 hr. culture	"	0	0	0			22 "	+	++++
18	Passively immune	10 cc. N.Y.S serum intravenously	13,000,000, 18 hr culture	"	30	750	1920			22 "	+	++++
19	Control	None	13,000,000, 18 hr. culture	"	+ (Contam.)	10,800	∞			22 "	No autopsy	
20	Passively immune	10 cc. N.Y.S. serum intravenously	9,000,000, 18 hr. culture	"		135	4920	1060		45 "	+	++
21	Passively immune	10 cc. N.Y.S. serum intravenously	4,500,000, 18 hr. culture	"		56	510	10	640	72 "	0	++
22	Passively immune	10 cc. N.Y.S. serum intravenously	1,000,000, 18 hr. culture	"		80	265	0	780	72 "	0	++++
23	Control	None	1,000,000, 18 hr. culture	"		1740	12,280	41,600		45 "	+	++
24	Passively immune	10 cc. N.Y.S. serum intravenously	5,000,000, 18 hr. culture	"		0	175	0		70 "	0	+++
25	Passively immune	10 cc. N.Y.S. serum intravenously	5,000,000, 18 hr. culture	"		0	5	0		24 "	0	++++
26	Control	None	5,000,000, 18 hr. culture	"		11,360	16,280			Hemorrhage 46 hrs.	+	+

TABLE I—*Concluded.*

No.	Type	Method of immunization	Infecting dose	Site of infection	Result of blood cultures					Duration of life	Post-mortem heart blood culture	Meningeal reaction
					1–1½ hrs.	2½–3 hrs	5–5½ hrs	24 hrs.	48 hrs			
27	Passively immune	10 cc. N.Y.S. serum intravenously	6,000,000, 18 hr. culture	Ear vein		0	5	0		Lived		
28	Passively immune	10 cc. N.Y.S. serum intravenously	6,000,000, 18 hr. culture	" "		0	0	0		"		
29	Control	None	6,000,000, 18 hr. culture	" "		4920	7640	∞		70 hrs.	+	
30	Actively immune	12 injections, 1 cc. vaccine, intravenously	3,000,000, 18 hr. culture	Cistern		0	15	0		Lived		
31	Control	None	3,000,000, 18 hr. culture	"		27,000	∞			27 hrs.	+	+
32	Passively immune	10 cc. N.Y.S. serum intravenously	7,200,000, 18 hr. culture	"	0	60	700			48 "	+	++++
33	Passively immune	10 cc. N.Y.S. serum intravenously	4,800,000, 18 hr. culture	"	5	3640	2680			48 "	+	++++
34	Control	None	2,400,000, 18 hr. culture	"	1400	10,200	18,900			72 "	+	+
35	Normal	"	6,000,000, 18 hr. culture + fibrinogen	"	0	0	0			96 "	+	+
36	"	"	6,000,000, 18 hr. culture + fibrinogen	"	0	0	0			26 "	+	+++
37	"	"	6,000,000, 18 hr. culture	"	2400	∞	∞			22 "	+	+

injections each of heat-killed Type I pneumococci. Each injection consisted of the quantity of organisms contained in 1 cc. of 12 hour broth culture, centrifuged and resuspended to original volume in saline. Agglutination titers in these actively immunized rabbits did not exceed 1:5 after one series of injections, nor 1:20 after two series. Of about 80 animals examined, only one showed agglutinins in the spinal fluid. In a small series of rabbits actively immunized by both intravenous and intracisternal injections of killed Type I pneumococci, no agglutinins were present in the spinal fluids when examined 1 week after the final injection. It would appear, therefore, that any restraint observed in the progress of the pneumococcal meningitis locally in partially immune animals is not due to agglutinating antibodies in amounts capable of titration in the spinal fluid at the time of infection.

A considerable number of animals was immunized in the above manner. Subsequent infection was by direct inoculation of organisms in the cisterna magna. The preliminary experiments on restraining the septicemia were performed with an 18 hour broth culture, producing a growth of about 250,000,000 per cc. In all but the initial experiments the dosage was determined by the use of the Petroff-Hausser counter. Bleedings were made from the ear vein at varying intervals after infection, and the number of colonies per cc. of blood counted. A control non-immune rabbit was used for comparison for all doses of organisms. The results of immunization in controlling the development of the septicemic spread from the meninges are evident in Table I.

In all the experiments summarized in the first table, the organism used was type-specific and capsule-forming, and produced only the smooth variety of colony. In view of the recently developing interest in pneumococcus variants (R forms), it was considered desirable to test out such R forms for meningeal pathogenicity. The R form used was obtained from Dr. Avery and was derived experimentally from the same type-specific strain employed above. It was developed from a single cell colony.

Rough Type I pneumococci were injected intracisternally in eleven rabbits. Some were normal rabbits; others had recovered from *Streptococcus scarlatinæ* meningitis. In all, a transient bacteremia

TABLE II.

No.	Type	Dosage Type I R	Results of blood cultures 1–1½ hrs	3–3½ hrs	5–5½ hrs.	24 hrs.	Duration of life	Autopsy cultures Heart	Cistern	Meningeal reaction
38	Normal	100,000,000, 18 hr. culture, cistern	1020	3760	800		24 hrs.	0	+	+
39	"	50,000,000, 18 hr. culture, cistern	300	35	25	0	Lived			
40	"	20,000,000, 18 hr. culture, cistern	5	15	5	0	"			
41	"	100,000,000, 18 hr. culture, cistern	0	160	525		24 hrs.	0	+	+
42	*Strep. scarlatinæ*; recovery	100,000,000, 18 hr. culture, cistern	5	5	5	0	Lived			
43	*Strep. scarlatinæ*; recovery	100,000,000, 18 hr. culture, cistern	0	30	0	0	48 hrs.	0	+ Very rare	+
44	*Strep. scarlatinæ*; recovery	250,000,000, 18 hr. culture, ear vein	3120	320	300	0	Lived			
45	*Strep. scarlatinæ*; recovery	100,000,000, 16 hr. culture, cistern	220	3640	35,280	0 (Paralysis)	14 days (Pneumonia)	0	0	0
46	Normal	100,000,000, 16 hr. culture, cistern	300	13,320	1740		24 hrs.	+	+	+
47	*Strep. scarlatinæ*; recovery	100,000,000, 16 hr. culture, cistern	960	440	225	0	7 days (Enteritis)	0	0	±
48	Normal	100,000,000, 16 hr. culture, cistern	240	215	65	0	78 hrs. (Pneumonia)	0	0	+

resulted (Table II). Only once, did the latter persist for 24 hours. Large doses of organisms were used, usually 100,000,000. This dose frequently killed. The mechanism of death is not clear. Few organisms could be detected in cistern fluid smears at death, but they were mostly phagocyted. The edema and congestion of the lungs were quite marked. If death did not occur within 24 hours, recovery was the rule. In a series of eleven animal passages, no reversion to a smooth colony type was observed.

It is obvious from consideration of Table I that active or passive immunization of animals previous to cisternal infection results in a retardation of septicemia. Where, exactly, this retardation occurs is difficult to determine on account of the multiplicity of factors involved, and on account of these variable factors, after a given degree of immunization the extent of the septicemia following intracisternal infection is irregular. The appearance of organisms in large numbers in spinal fluids is delayed. This retardation was especially notable in later experiments on treatment, where frequently, after an injection of upwards of 3,000,000 organisms intracisternally, the 19 hour cisternal fluid showed rare pneumococci only, and where in one instance spontaneous recovery of an untreated immune rabbit occurred, when all treated animals of that group died. It is likewise evident in early experiments (Table I), that in actively immune animals much larger infecting doses are required to produce a fatal meningitis. So far as was determined, this local meningeal retardation does not depend upon the presence of agglutinating antibodies in the fluid at the time of infection, although their ultimate influence is not beyond the realm of possibility, since following the cisternal injection, choroid plexus alteration may result in some diffusion of agglutinating antibodies present in the blood, or of opsonins (7). We have not done any opsonic studies on spinal fluids of immune animals.

In partially immune rabbits pneumococcal meningitis is occasionally abortive, but it would appear that in the meninges, doses of organisms will produce progressive disease and also eventual septicemia, whereas several times the number of diplococci injected intravenously will produce nothing at all. Therefore it may be assumed that the meningeal spaces constitute to some, and probably to a considerable extent, a non-immune locus, where organisms in numbers insufficient to

produce systemic disease may multiply, pass the filtration mechanism in great numbers, and break down the systemic resistance.

After the series of preliminary experiments outlined in Table I, we determined to try the effect of antisera in the control of the meningeal disease. Not much hope was entertained, in view of the severity of the process, but it was thought that if any evidence at all indicated benefit from serum therapy in so extremely susceptible an animal as the rabbit, there would be some justification in advocating its utilization. In the majority of instances, the concentrated antibody solution prepared for us by Dr. Lloyd Felton, of the Harvard Medical School, was used. According to Dr. Felton's titration, the samples employed by us contained 50 and 3000 protective units per cc. We must acknowledge that so far as beneficial therapeutic results are concerned, we were unable to see any difference in the two strengths of antibody solutions, nor could we detect that either differed appreciably from the Type I antipneumococcus serum prepared in the New York State laboratories. It is impossible to express results of therapeutic experiments in tabular form, and since it is not desirable to include all protocols, a short series only of the latter is included. It may be emphasized here that, whereas for convenience in planting and timing cultures, an 18 hour growth was used in preliminary studies of the septicemia, in therapeutic experiments a 6 hour culture was used. The first culture had obviously gone beyond its developmental peak, while the second was still in an active growth phase. It became apparent early that the second culture was much more active and that with given immunization a smaller dose of the 6 hour culture would duplicate the results of a larger 18 hour culture. It was likewise noted that the apparent vigor of the culture, as expressed by actual growth per cc. in a given number of hours, was also very important. Since the actual dosage of organisms in each instance was counted, we are forced to conclude that there must have been either an actual variability of potency in the individual cocci under unfavorable growth conditions, or possibly that the total constituency of the culture varied, cultures giving poor growth containing more damaged or dead cocci. These factors may be of minor importance in influencing ultimate fatal disease in normal rabbits, but may play a considerable rôle where partial immunity has been established. Then again, there

is an uncontrollable individual variation in animals; given a certain dosage of immune serum and a certain known cisternal dosage of pneumococci from the same culture, results will vary. These varying factors are shown in the following few representative protocols.

Illustrative Protocols.

Rabbit A.—Active immunity established by intravenous and intracisternal vaccine Immunization completed December 4, 1926. December 14, 1926, 4.00 p m., ether, cistern puncture, clear fluid, injected 17½ million Type I pneumococci. Good recovery. December 15, 9 30 a.m , temperature below 92°. Cistern puncture, rare cells but a massive, diffuse overgrowth of pneumococci No phagocytosis Injected 1 cc Felton 50 unit antibody solution intracisternally, and 5 cc. antipneumococcus serum intravenously. Placed on electric pad Temperature recovery to 100.2°. Dead 2 30 p m. Autopsy: Brain is pale, meninges slightly opalescent. Cisternal fluid, instead of rare cells observed previous to treatment, contains, on smearing, from 60–100 polymorphonuclears per oil immersion field Very active phagocytosis of pneumococci; free organisms largely agglutinated. Culture: massive growth; heart blood: no growth. Microscopically: fibrinopurulent exudate over cerebrum, cerebellum, medulla, and cord; exudate also involves dura, choroid plexuses, cord septa, sheaths of penetrating vessels Large number of pneumococci Phagocytosis confined essentially to region of cisterna. Moderate superficial encephalitis. Note: an example of massive proliferation of pneumococci in the meninges, no terminal septicemia, an initial poor cellular reaction in meninges, greatly improved by antibody injection, and induction of marked phagocytosis.

Rabbit B—Same immunization and infecting dosage as in Rabbit A. Infected same day with same culture 18 hours after infection, temperature 103 2°, sluggishness, fine tremors, 22 hours, hind limbs paralyzed Cistern puncture under ether; cells 4–12 per o. i. f., moderate number of cocci, no phagocytosis. Injected 1 cc. 50 unit antibody, cisternally, and 5 cc. antiserum, intravenously. Temperature fell to 95°. 24 hours, second cistern puncture; cells 20–100 per o. i. f.; rare agglutinated cocci First culture, massive growth; second, few colonies only. 42 hours· cisternal fluid contained large numbers of cocci. Injected 2 cc antibody, with symptoms of pressure. 66 hours: fluid as above Injected 1.5 cc. antibody. Death in 2 hours. Autopsy. Similar to Rabbit A, save that the lumbar cord is essentially gangrenous. Cisternal cell number again markedly increased (60–100 per field); agglutination and phagocytosis of cocci. In addition to usual microscopic findings, considerable periarteritis of larger vessels of cord meninges, purulent ventriculitis of third and lateral ventricles, abscesses of choroid plexuses of fourth and lateral ventricles, and ingrowth of cocci into wall of lateral ventricle. Summary: Same immunization and dosage, but a considerably lesser initial process. Repeated treatments always

resulting in increased cellular reaction and phagocytosis, but in the interim between treatments great augmentation of cocci from regions untouched by treatment. Heart blood: no growth.

Rabbit C.—Immunization as in the two preceding. December 16, 1926, 4 00 p.m., ether, cistern puncture, clear fluid. Injected 12 million Type I pneumococci, 7 hour culture. 18 hours later, temperature 106.5°. Cisternal fluid opalescent, cells 10–20 per o. i. f.; only occasional clusters of diplococci; culture slight growth. By vital staining cells 64 per cent polymorphonuclears, 34 per cent monocytes, 2 per cent small lymphocytes. Injected 1.5 cc. antibody solution. 23 hours, lumbar and cistern punctures; leucocytes increased; very few phagocytic cocci; none free. Cultures: no growth. Injected 1 cc. antibody in cistern and in lumbar cord. No further treatment and an uneventful recovery. Summary: Delayed development of meningeal infection with few organisms in fluid at time of treatment. Rapid sterilization of a mild process. Recovery not necessarily due to treatment since immunization may have played the more decisive rôle.

Second animal of this group, similarly treated, developed a massive growth of cocci and died in 41 hours with only moderate meningeal reaction and a slight septicemia These two rabbits emphasize the individual factor. A control, actively immune, untreated rabbit died in 41 hours with severe septicemia and a good meningeal reaction.

Rabbit D.—December 13, 1926, 9 50 a.m , 10 cc. Type I antipneumococcus serum, ear vein. 4.10 p.m., ether, cistern puncture, clear fluid, injected 3,000,000 Type I pneumococci, 7 hour culture. 18 hours after infection temperature 106.4°. Cisternal puncture; fluid slightly opalescent; cells very rare and only occasional cocci. Culture slight growth. Injected 1 cc. 50 unit antibody. 24 hours after infection second cistern puncture; fluid abundant, almost purulent; polymorphonuclears about 100 per o. i. f.; no cocci seen; culture negative; 1 cc. antibody injected. 42 hours, temperature 105.4°; active. 66 hours: appears slightly ataxic and has a head nystagmus. Spinal fluid culture negative. 5 days: temperature 104.5°; improved. 7 days: unchanged. 10 days: found dead. Autopsy: Spinal cord and brain pale, meninges opalescent. Cisternal fluid seropurulent, containing 50–100 polymorphonuclears per o. i. f. and a diffuse overgrowth of pneumococci. Marked congestion and edema of lungs. Culture of heart blood, no growth. Usual histology of fibrinopurulent meningitis. Summary: Low grade initial process in passively immune rabbit; culture of cisternal fluid twice negative after antibody injections. Final recrudescence of meningeal infection leading to delayed death at 10 days.

A second rabbit of this group, similarly immunized and infected, developed a massive growth of pneumococci within the first 18 hours, with essentially no cellular reaction. The latter was greatly improved by 1 cc. of antibody and at death, 3 hours later, there were large numbers of leucocytes and phagocytosis of 50 per cent of the organisms in the cisternal fluid. Blood cultures gave moderate growth.

The passively immune, untreated control died of meningitis and septicemia in 36 hours.

Rabbit E.—December 20, 1926, 10.00 a.m., 10 cc. Type I antipneumococcus serum ear vein; 4.00 p m , 3,000,000 Type I pneumococci intracisternally. 18 hours: temperature 106 2°; active. 42 hours: temperature 106 3°; sluggish; diagnostic cistern puncture; cells rare; pneumococci very rare; culture positive; blood sterile. 66 hours: temperature 105.4°; sluggish; marked ataxia and weakness; diagnostic cistern puncture; essentially no change in fluid picture. 88 hours: dead. Petechial hemorrhages over cerebellar hemispheres; meninges opalescent, brownish, gelatinous looking exudate at base; cord not grossly remarkable; cisternal fluid smear shows about 100 leucocytes per o. i f. and a large diffuse overgrowth of diplococci. Practically no phagocytosis Microscopically: a thick fibrinopurulent meningitis. Summary: A markedly delayed initial process with essentially no early cellular reaction, a terminal extensive leucocytosis with little phagocytosis and a diffuse coccal overgrowth.

Of this group two animals were treated; both had mild initial processes at 18 hours and were lavaged from lumbar to cistern with antibody solution. One died at 88 hours with a pneumonia from which Type I pneumococci were isolated; at death both spinal fluid and heart blood gave no growth. The other animal recovered Although this effect would appear to indicate favorable results of treatment, in our opinion it means nothing. There are too many variable factors involved to make correct analysis possible.

In recovered rabbits, various postmeningitic signs may occur. Prominent among these are paraplegias, ataxia, nystagmus, and prolonged opisthotonus. With the exception of the paraplegias, there is a strong tendency to amelioration of these conditions

Whereas in small series of three to five rabbits, similarly immunized and infected, controls may die and treated animals recover, in one instance the reverse has been true, four treated animals dying, and one untreated recovering This occurrence only goes to prove the impossibility of evaluating results when such variable factors are present. Individuals react differently after the same immunization; there is a very narrow zone between a dose of organisms which in a partially immune animal may produce a massive growth of cocci in the meninges with or without septicemia, and a dose which may permit spontaneous recovery If we take length of life as an index of value of therapy, it is apparent that the treated animals dying, lived an average of 24 hours longer than the controls; if we consider the presence or absence of septicemia, it becomes evident that 28 per cent of animals treated locally and systemically subsequent to infection had positive blood cultures at autopsy and that 70 per cent of partial immunes with no subsequent treatment had terminal septicemia. Length of life in treated animals is difficult to interpret on account of a high percentage of traumata with repeated punctures; working space is very small in the rabbit, and the needle permitting satisfactory lavage is of course disproportionately large. Reac-

tion to intracisternal injection of antibody solution is often pronounced. We may summarize it in the following protocol.

Rabbit F.—Actively immune; uninfected; temperature 102.5°. Ether; cistern and lumbar puncture; clear fluid. Lavage with 2 cc. antibody solution, strength 50 units per cc. Immediate ether recovery. Within 10 minutes, prostration, tremors, chill, progression movements of all four limbs, rapid, shallow respiration, temperature 95.7°, falling in 2 hours to 92°. Following morning slightly sluggish, temperature 99.5°. Subsequent uneventful recovery.

In one instance the reaction did not begin until 1½ hours after injection of antibody. Samples of antibody containing no tricresol produced at times similar effects. These reactions may be duplicated by trauma to medulla, but frequently no trauma could be verified at autopsy. In the uninfected animal intrathecal antibody solution causes a slight transient polymorphonuclear reaction, rapidly passing over to an exudate containing small lymphocytes and endothelial leucocytes.

Pathology.

The microscopic appearances of the pneumococcal meningitis have been studied in all the animals. The pathology is distinctly a function of immunization, dosage, and treatment. At one extreme one has the non-immune rabbit infected *via* the cisterna. The tendency in this animal is for the cerebrospinal fluid to be turned into a veritable culture medium for pneumococcus (Fig. 3); the pia-arachnoid is filled with a massive sheet of pneumococci; the choroid plexuses contain them in great numbers; they invade the Virchow-Robin spaces in solid masses (Fig. 4); they multiply within the ventricles. In this form of disease there is very little reactional change in the meninges; death is always with marked septicemia usually complicated by edema and congestion of the lungs. In a partially immunized rabbit death is delayed, and time is allowed for a vigorous cellular reaction in the meninges; this takes the form of a thick, fibrinopurulent exudate, involving the meninges of all regions, cord, cerebellum, convexity, base and choroid plexuses. Its intensity is variable. Pneumococcal proliferation may be restrained at first, but eventually usually reaches an extreme degree, the cocci lying in solid masses between reacting cells (Fig. 5); there is little phagocytosis. If there is intrathecal treatment, an increased leucocytosis, with active phagocytosis, agglutination, and thread reaction result (Figs. 6–8). Phagocyted cocci stain supravitally with neutral red. These phenomena are but transitory and

within a few hours there is a tendency for cells to diminish and for the diffuse growth of organisms to be resumed. This constant renewal of pneumococci originates from agglutinated, unphagocytized clusters of pneumococci and particularly from regions untouched by treatment. It is evident that it is impossible to reach, by treatment, all regions in the rabbit, if the initial growth of bacteria is at all severe. Exudate with pneumococci may be found in the central canal of the cord, between fibers of issuing nerve trunks (Figs. 9 and 10), or in spinal ganglia (Fig. 11). There may be empyema of the central canal of the cord, with destruction of the ependyma and involvement of either the cornua or funiculi (Fig. 12). Pneumococci in the sheaths of penetrating vessels are commonly observed. In any severe process, one is apt to find a superficial encephalitis (Figs. 13 and 14), or else foci of softening and invasion by pneumococci (Fig. 15). Periarteritis is common (Fig. 16). Purpuric hemorrhages may occur either in the meninges or deep in the brain or cord substance (Fig. 17). Death is almost always complicated by edema and congestion of the lungs. There may be a considerable fibrin deposit and if life is sufficiently prolonged, a lobular consolidation may result (Figs. 18–20). Pneumococci may be demonstrated in this exudate by Gram-Weigert staining.

SUMMARY AND CONCLUSIONS.

1. Fatal Type I pneumococcal meningitis may be produced in rabbits by intracisternal injection of pneumococci.
2. When organisms are of high virulence, the rabbit does not tend to localize them in the meninges, but an early septicemic process results. Death is septicemic rather than meningeal.
3. In such instances very little cellular reaction occurs in the meninges.
4. Active or passive immunization previous to intracisternal infection inhibits partially the septicemia and permits the development of reactional processes in the meninges.
5. The immunization likewise retards the meningeal disease, but multiplicity of factors prevents us from stating precisely to what this retardation is due. It is not correlated with the presence of agglutinins in the spinal fluid at the time of infection.
6. The rapidity of production of meningitis is influenced by the

phase of growth of the culture used, and likewise by the growth activity of that culture.

7. To some extent in the partially immune rabbit the meningeal spaces constitute a relatively non-immune reservoir, constantly feeding the blood stream and breaking down systemic resistance.

8. Intrathecal serum treatment causes rapid agglutination and phagocytosis of pneumococci, and has very rarely, possibly, resulted in cure. Essentially no phagocytosis occurs in the absence of immune serum.

9. Phagocyted pneumococci stained supravitally take up the neutral red stain and are therefore probably injured.

10. The treatment employed subsequent to infection only slightly prolongs life in the majority of cases. It does retard septicemia.

11. The treatment improves the cellular reactional processes in the meninges.

12. A study of the pathology of rabbit pneumococcal meningitis shows that the location of pneumococci precludes complete contact with serum introduced intrathecally, and that these locations provide isolated foci, from which organisms may reinfect the meningeal spaces as rapidly as they are removed by lavage or antibody injections.

13. In recovered rabbits postmeningeal symptoms, weakness, ataxia, nystagmus, and paralyses arise.

14. In our opinion there is some objective evidence of benefit of serum therapy. The rabbit is too susceptible, however, and conditions too artificial to admit of definite conclusions.

15. Rough Type I pneumococci introduced in large quantity cisternally may kill and may be recovered 24 hours after infection, from spinal fluids. In a series of eleven passages, no reversion to smooth type occurred. In all animals injected with rough forms, transient bacteremia resulted.

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EXPLANATION OF PLATES.

Plate 11.

Fig. 1. Non-immune rabbit. Pneumococci in lung capillaries. Pulmonary edema. Gram-Weigert stain. × 1000.

Fig. 2. Same animal stain, and magnification. Pneumococci in capillary of renal medulla.

Fig 3. Spinal fluid smear Shows type of fluid found in a non-immune rabbit or in one partially immune, which has developed a massive overgrowth of cocci previous to treatment. × 1000.

Fig. 4 Non-immune rabbit Solid masses of pneumococci in perivascular sheath of penetrating cortical vessel Gram-Weigert stain. × 1000.

Plate 12.

Fig. 5. Massive growth of pneumococci in partially immune treated rabbit. Slight phagocytosis. Section of cord far distant from region of treatment. Gram-Weigert stain. × 1000.

Fig. 6. Agglutination and vigorous phagocytosis in exudate of treated animal. × 1000.

Fig. 7. Thread reaction in agglutinated cocci after treatment. × 1000.

Fig 8. Phagocytosis of pneumococci in exudate of treated animal. The same animal as in Fig 5, but from region near location of treatment. Gram-Weigert stain. × 1000.

Plate 13.

Fig. 9. Polymorphonuclear invasion of spinal nerve trunk. × 260.

Fig 10. Growth of pneumococci between fibers of spinal nerve trunk. Non-immune rabbit. Gram-Weigert stain. × 1000

Fig. 11. The same animal, stain, and magnification. Pneumococci in a spinal ganglion.

Fig. 12. Cord abscess (pneumococcal) in a partially immune rabbit, an extension from empyema of central canal of cord × 150.

Plate 14.

Figs. 13 and 14. Superficial encephalitis Partially immune rabbit × 260.

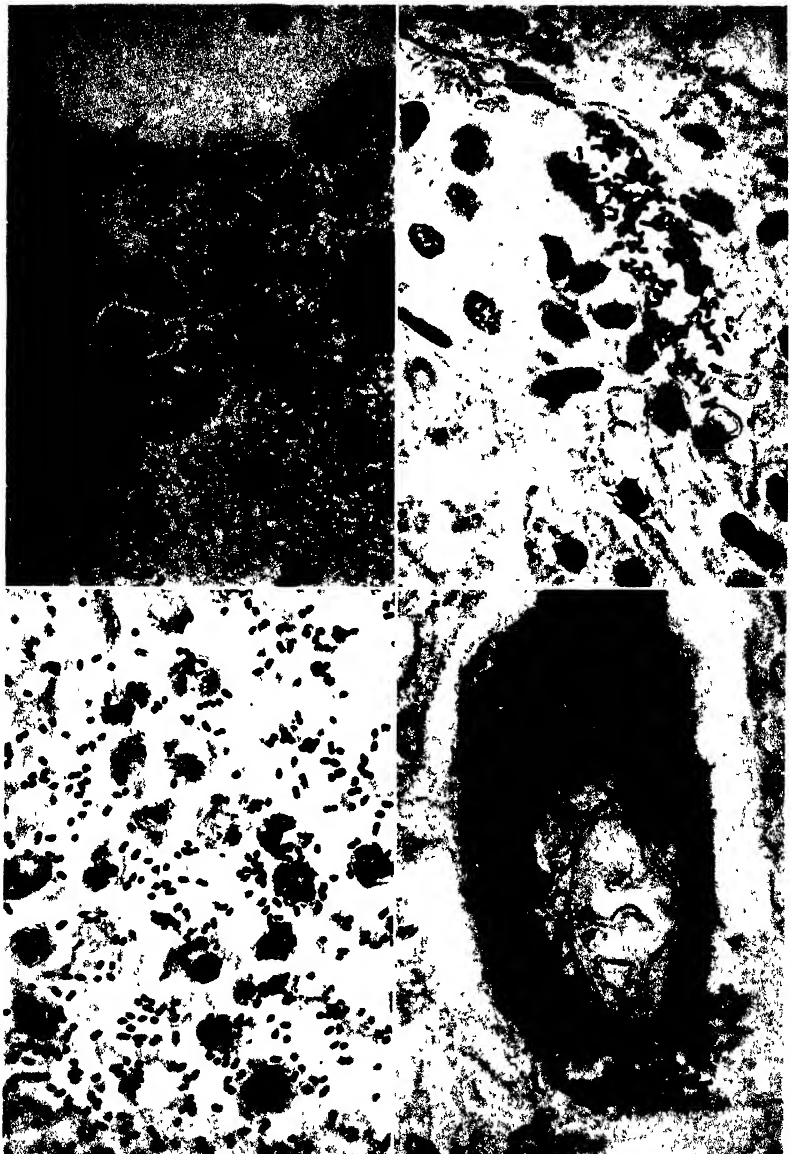
Fig. 15. Pneumococcal invasion with softening of wall of lateral ventricle. Treated animal dying at 21 hours. Gram-Weigert stain. × 1000

Fig. 16. Periarteritis, meningeal cord vessel. Partially immune rabbit. × 260.

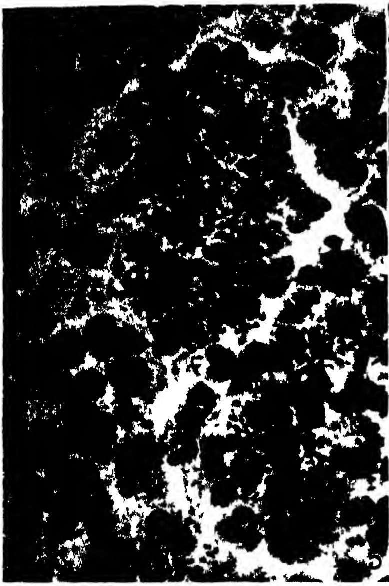
Plate 15.

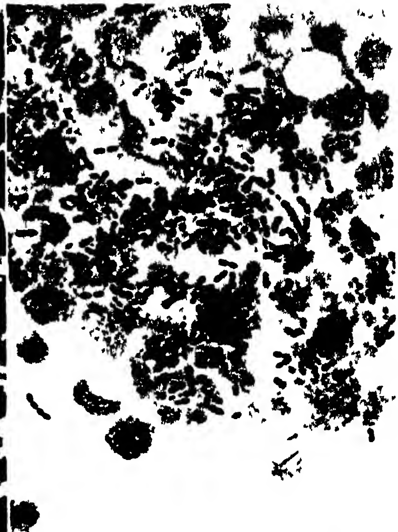
Fig. 17. Hemorrhagic lesion, gray matter of cord. Partially immune rabbit dead 28 hours after infection. × 110.

Figs. 18 to 20. Pneumococcal pulmonary lesions as observed in partially immune rabbits. × 50.



(Stewart Pneumococcal meningitis I)





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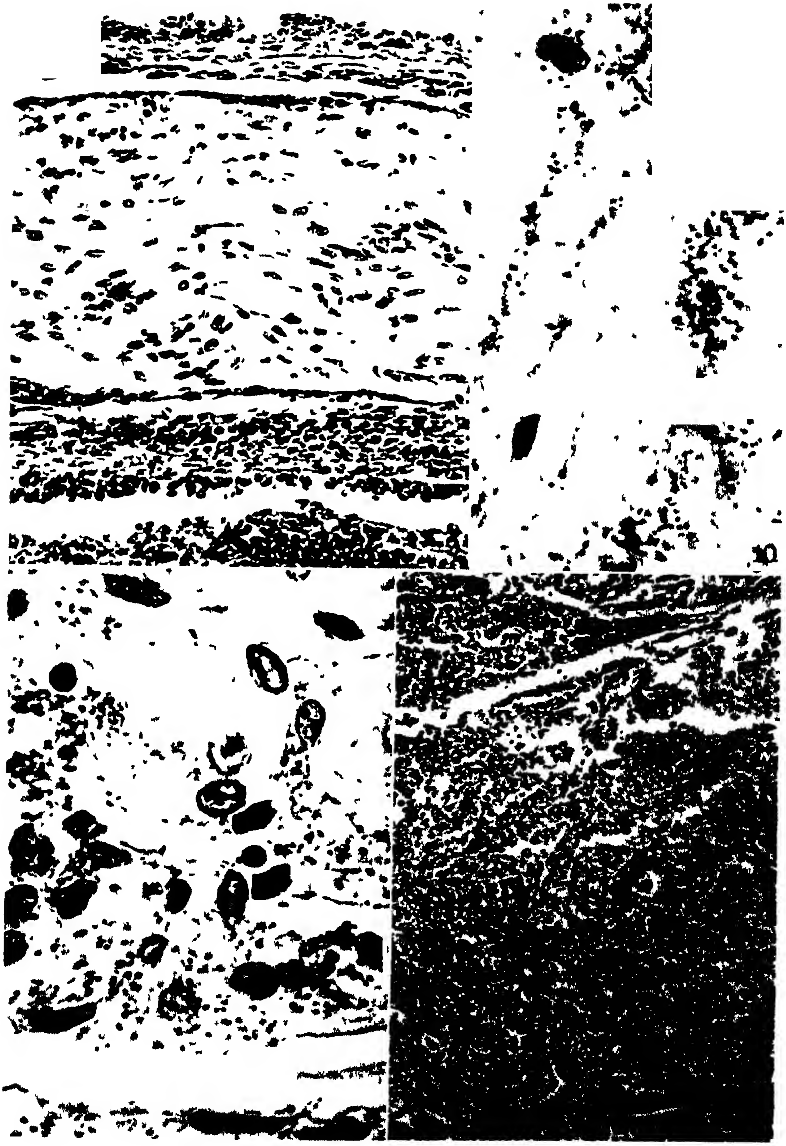

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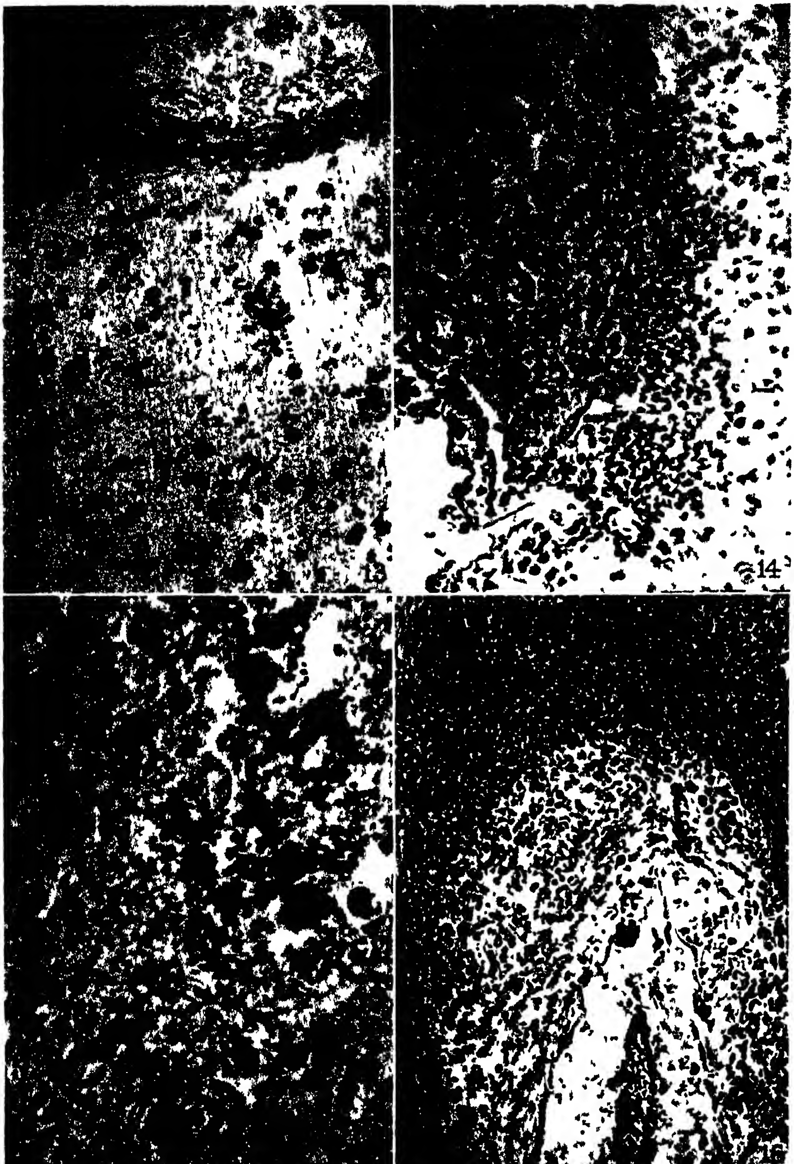

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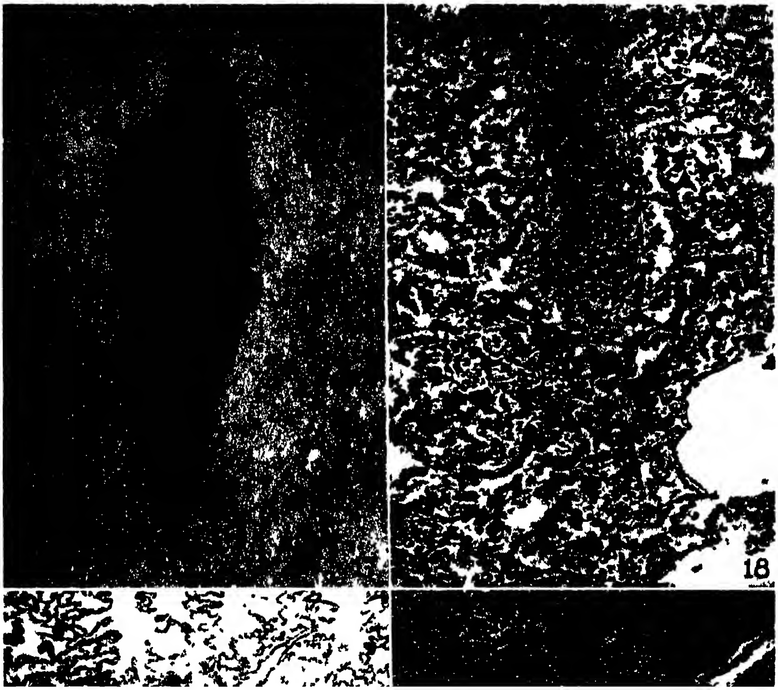

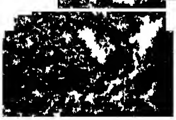

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[Reprinted from The Journal of Experimental Medicine, September 1, 1927, Vol. xlvi, No. 3, pp. 409–427.]

LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

II. The Production, Pathology, and Treatment of Type I Pneumococcal Meningitis in Dogs.

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Plates 16 to 21.

(Received for publication, June 12, 1927)

In the previous paper (1) the production, pathology, and attempted treatment of Type I pneumococcal meningitis of rabbits were discussed. Reasons were presented explaining why the rabbit is a very unfavorable animal for the study of this disease, which may be briefly summarized as follows: In a normal rabbit, septicemia, following intracisternal infection with Type I pneumococci, is invariable, rapid, and massive, and septicemic death occurs before there is any reactional localization in the meninges; consequently, to produce meningeal localization, complete or partial, a previous immunizing treatment is necessary. Following this treatment, however, there is a considerable variation in the intensity of the meningeal infection, making it impossible to gauge properly the dosage of organisms which will produce fatal disease, but which will not immediately overwhelm the animal's resistance. Even if the disease intensity is properly regulated, treatment is unsuccessful on account of both the limited working space and the widespread localization of organisms in regions impossible to reach either by immune serum or lavage.

In view of these facts, the rabbit was abandoned as an experimental animal and recourse was had to young dogs. The dog is, as we know, relatively resistant to systemic pneumococcal infection, although marked variations even here are encountered. In the meninges, however, a type of disease may be produced which progresses toward fatal termination, if cultures of suitable virulence are used, and one which quite closely approximates pathologically the human pneumococcal

meningitis. To produce rapid progressive meningitis with any degree of assurance that the disease will assume a fairly characteristic course from day to day, the infecting dose must be reasonably standardized. In the present study the dosage of organisms has been accurately counted by means of a Petroff-Hausser bacterial counter. Not only should the numbers of infecting pneumococci be known, but the culture should be standardized both as to hours of growth and as to quantity of growth. A culture should be chosen which is well along on the upward limb of the growth curve. We have employed 6 hour cultures. It must be a culture which has given a good growth. There is a marked difference between the degree of 18–24 hour infection produced by a given number of pneumococci from a 6 hour plain broth culture which has produced a low total growth, *i. e.*, a growth ranging from 150–225 million per cc., and that produced by the same number of organisms from a 6 hour 0.2 per cent dextrose broth culture which is producing a growth of upwards of 700 million per cc. Unless these differences are recognized, conclusions are of little value. Use of late (24 hour) cultures may lead to very deceptive results. Even with these cultural conditions standardized, there is an uncontrollable individual factor which influences the rate of establishment of progressive disease; every animal is an individual and must be so handled. Whereas we hesitate to interpret the anatomic and therapeutic results of others, we are inclined to believe that the findings of Idzumi (2) were the result of the use of a culture of low virulence. Idzumi studied pneumococcal meningitis in dogs. This investigator used enormous doses of organisms, from 1–2 cc. of a suspension secured by centrifuging 10 cc. of a 24 hour culture of low mouse virulence. Although the dogs died, the pathologic changes were scarcely more than those of hyperemia. In our own study the disease has been regularly produced with approximately 1 cc. of a 1:100 dilution of 6 hour culture. It would seem that Kolmer's (3) successful therapy, *i.e.*, lavage from one or both lateral ventricles to the cisterna magna with physiological saline or Ringer's solution, cannot be interpreted as the result of other than feeble infection with cultures well beyond the growth peak and full of non-viable organisms. The lavage was most incomplete, since the entire cord, base, and convexity were untouched by the saline. Kolmer reports, however, that at the time of lavage

the spinal fluids were always purulent and contained myriads of pneumococci, making it more difficult for one to understand how lavage from *one* lateral ventricle to the cisterna magna could have appreciably influenced the process, since in our own experience the other ventricle would always have been an untouched focus in a disease of the intensity he describes. Bull (4) observed meningitis developing in the course of pneumococcal septicemia in dogs. His organism was not typed. Bull noted some spontaneous recoveries.

EXPERIMENTAL.

Apparently in control dogs, infected intracisternally, death may occur in one or more of three ways. A dog may develop a fairly rapid proliferation of pneumococci in the meninges with a constant large feeding of organisms into the blood stream, and die of bacteremia. If a culture is of low potency due to poor growth conditions, a meningeal process of such intensity may result that a very marked cellular reactional process occurs, with pneumococci present in relatively small numbers. In such cases death appears to be "reactional." Thirdly, the proliferation of organisms within the meninges may attain enormous proportions, and brain and cord may be covered with a thick, gelatinous layer, consisting mainly of heavily encapsulated pneumococci, but with relatively moderate cellular reaction. In the second and third instances, death may be due, in part at least, to the mechanical obstruction of the meningeal spaces. The actual mechanism of death from pneumococcus infection is always rather obscure. Three protocols may be introduced to illustrate these points.

Dog 12.—Female hound; weight 5.5 kilos. March 23, 1927, 4.10 p.m., mor phine gr. 1/5; ether; cistern puncture; clear fluid. Injected 6,500,000 Type I pneumococci (6 hour dextrose broth culture, growth 1,100,000,000 per cc.). Good recovery. 19 hours later, lethargic, weak, irritable, sicker than usual at this stage. 23 hours, morphine gr. 1/5; death in 10 minutes. Autopsy: Brain and cord injected, edematous; cisternal fluid cloudy; marked grayish yellow opacity along sulci of convexity and at base. Smears from the usual regions show on an average 20–30 pneumococci per oil immersion field and 30–60 cells, mostly polymorphonuclears. Viscera negative. Culture from heart's blood gave massive confluent growth. A death mainly due to septicemia. Microscopically, however, there was a marked diffuse fibrinopurulent leptomeningitis.

Dog 3.—Female hound; weight 7.5 kilos; February 9, 1927, 4.20 p.m., ether,

cistern puncture, clear fluid. Injected 15,000,000 Type I pneumococci (7 hour plain broth culture giving poor growth). Good recovery.

23 hours—temperature 103°. Sluggish; refuses food.

43 hours—temperature 100.2°. Unchanged; 100 cc. saline intraperitoneally.

67 hours—temperature 103°. Sleeps most of time; able to stand; 75 cc. saline intraperitoneally.

93 hours—temperature 102.2°. Able to stand leaning against cage; weak; stiffness of neck; 75 cc. saline.

5th day—temperature 102°. Very irritable.

6th day—temperature 103.2°. Irritable; chills; tremors.

7th day—temperature 99.8°. Prostrate; hypersensitive; tremors; incontinence of urine and feces; 100 cc. intraperitoneal saline.

8th day—dead. Autopsy: Upon opening spinal cord and cranial cavity, meninges are opaque and pinkish yellow; purpuric blotches in region of medulla. Cord dura is very tense; on slitting it, thick, grayish or greenish yellow pus exudes; this is generalized in distribution, but most abundant in lower medullary, upper cervical, and region of lumbar enlargement. Smears show thick masses of polymorphonuclear leucocytes and rare endothelials. Only occasional diplococci, all extracellular. Over the cerebellar vermis and adjacent portions of cerebellar hemispheres is thick pus. A smear shows a similar picture to that from cord. The base of the medulla and the pons is coated with thick, greenish yellow pus (Fig. 1). Much less exudate over convexity, and pneumococci are rare. The lateral ventricles are dilated and filled with thick pus, and the walls appear eroded In the ventricular pus cocci are more numerous, but still do not reach the usual proportions; are free and in small agglutinated clusters. Essentially no phagocytosis. Heart's blood cultures are negative.

Summary: Infection with culture giving poor growth; delayed death; enormous reactive process; few pneumococci at autopsy. Suggests a "reactional" death. Histologically a very marked meningitis, superficial encephalitis and myelitis.

Dog 7.—Male fox terrier; weight 6 kilos. March 9, 1927, 4.05 p.m., ether; cistern puncture; clear fluid. Injected 7,000,000 Type I pneumococci (6 hour dextrose broth culture, growth 700 million per cc). Good recovery.

18 hours—temperature 103.5°. Stands, wags tail, refuses food and water.

42 hours—temperature 103.4°. Semiprostrate; 100 cc. intraperitoneal saline.

66 hours—very irritable; unable to stand.

90 hours—dead. Autopsy: Fourth ventricle distended with thick, gelatinous material; cord meninges and meninges over convexity and base tense, opaque, filled with similar yellowish gelatinous deposit. Lateral ventricles contain thick pus. Smears from all regions show numerous pus cells, but an incredible number of pneumococci (Fig. 2), the latter really making up most of the gelatinous material. Essentially no phagocytosis. Both cerebrospinal fluid and heart's blood give abundant growth.

At this point, the observation should be emphasized that smears taken with a loop from different regions of brain or cord meninges give rather deceptive results so far as numbers of exudative cells are concerned. Probably this is due to the fact that cells lie in fibrin meshes wherein they are held. Pneumococci may be picked up more easily and smears, so far as numbers of bacteria go, check fairly well with microscopic findings in sections.

Therapeutic Experiments.

After a few trials of the culture to determine a proper infecting dose, attempts were instituted to treat the animals. The first series consisted of three dogs, two treated and one control. The culture used was that described in the protocol of Dog 3 (above). This culture was of low virulence, producing a prolonged process in the control animal. The infecting dose consisted in 2,000,000 organisms per kilo of body weight; animals were injected intracisternally. It so happened that both treated dogs made uneventful recoveries, save that one had a residual deafness. These animals were treated at an early period in the work, really before we had had much experience in puncturing the cistern and lumbar subarachnoid space, and the daily progress of the disease was not sufficiently followed, nor was the condition of the fluid of one of the treated animals determined previous to treatment. The fluid of the other animal showed but 4–6 cells per oil immersion field, and but 4 diplococci in the entire smear; the culture was nevertheless positive. Treatments were in one instance with Felton's 3000 unit antibody solution, and in the other with unconcentrated antipneumococcus serum. The details of these treatments are not included, since from the behavior of the culture and of the control animal, we feel inclined to disregard this experiment. Suffice it to say that no subsequent test with really virulent culture has promised any duplication of this favorable result. The following protocol summarizes a more systematic study of the effect of treatment, and is introduced to show the behavior of a treated dog for which Dog 7, the animal described above in illustration of the massive pneumococcal overgrowth type of disease, was the control.

Dog 6.—Female bull terrier; weight 5 kilos. March 9, 1927, 3.50 p.m., ether; cistern puncture; clear fluid. Injected 7,000,000 Type I pneumococci (6 hour dextrose broth culture, growth 700 million per cc.). Good recovery.

18 hours—temperature 104.7°. Stands, wags tail, refuses food and water.

19 hours—ether; cistern puncture; lumbar puncture; lavage from lumbar to cistern with 20 cc. warm saline, followed by 15 cc. antipneumococcus serum; 5 cc 800 unit antibody solution intravenously. Cisternal fluid opalescent; looks like a suspension of cocci. Smears of this fluid as follows:

Before lavage—cells 20–30 per oil immersion field; myriads of cocci.

After 15 cc. NaCl lavage—cells rare; cocci about 80 per o. i. f.

After 20 cc. NaCl lavage—cells rare; cocci about 60 per o. i. f.

24 hours—temperature 103°; ether; cistern puncture; seropurulent fluid; drainage. Injected 5 cc. antipneumococcus serum. Smear: numerous pus cells; cocci largely agglutinated and phagocyted; still numerous, but less than at the end of the previous lavage. 75 cc intraperitoneal saline. Culture: confluent growth.

42 hours—temperature 101.6°. Weak, sluggish, able to stand. Ether, combined puncture; cloudy yellowish fluid; 20 cc. saline lavage, followed by 10 cc. antipneumococcus serum. Smear: before lavage, polymorphonuclears 30–150 per o i. f.; cocci not increased over previous smear. After completing lavage, both cells and organisms very rare. Culture: confluent growth.

66 hours—temperature 101.7°. Refuses to stand; lethargic; shortly after coming to laboratory a generalized convulsion with salivation and gnashing. Series of bloody stools. Ether; combined puncture; cells 3–60 per oil immersion field; cocci diminished; many look swollen; bacillary; coccoid. 10 cc. antipneumococcus serum.

90 hours—dead. Autopsy: Cord grossly normal; vessels in region of cerebellum and medulla injected; posterior convexity injected; fibrinopurulent material over anterior convexity and base. Thin seropurulent material in lateral ventricles Smears: cistern—cells 125 per o. i f.; cocci largely phagocyted. Cord—cells 50 per o i. f.; cocci very scanty. Anterior convexity—cells 200 per o. i. f.; cocci diffuse with only slight phagocytosis. Lateral ventricles—cells 30–40 per o. i. f.; cocci diffuse with marked phagocytosis Heart's blood culture—no growth. Viscera: acute intussusception (the cause of death). Microscopically the cord showed relatively little exudate, but the latter was very abundant over the convexity and cerebellum and in the fourth ventricle.

Summary: A very marked initial growth of cocci; clearing by repeated lavage; abundant phagocytosis of residual cocci following antiserum injections but a focus, especially over the anterior convexity, not appreciably reached by serum and thereby constituting a site for subsequent "reinfection" of other regions. Objectively a very decided benefit from treatment.

In view of the fact that it appeared quite possible to lavage certain regions of the central nervous system relatively free from organisms

and to reduce residual penumococci by the phagocytosis resulting from the injection of immune serum, it was considered desirable to extend, if possible, the field of action of the serum to those portions of the brain not reached by lumbar or cisternal injections. It was determined to treat the convexity by subdural serum injections, after trephining over the frontal lobes. The question arose as to whether one trephine was sufficient, or whether the falx constituted such a complete barrier that bilateral trephining was necessary. To settle this point a single frontal trephine was made just above the frontal sinus in a normal dog under ether anesthesia and 2 cc. of methylene blue was injected. The animal later received likewise 2 cc. intracisternally. The methylene blue injected frontally passed rapidly to the cisterna and with the additional cisternal injection was almost immediately recovered from the lumbar subarachnoid space. The dog was etherized after 1 hour. Staining of the brain was, as far as the convexity was concerned, sharply limited to the hemisphere of the injected side (Fig. 3). This made it obvious that to treat the infected convexity bilateral trephine openings were necessary. The following protocol summarizes one of the early animals treated by quadruple puncture.

Dog 10.—Fox terrier, female; weight 7.5 kilos. March 18, 1927, 3.15 p.m., ether; cistern puncture, clear fluid. Injected 8,400,000 Type I pneumococci (6 hour dextrose broth culture; growth 1,400,000,000 per cc.). Good recovery.

21 hours—temperature 99.8°. Sluggish; refuses to stand, will not drink. Ether; lumbar puncture; cells 3–5 per o. i. f.; no cocci seen, but culture positive Cistern puncture; cells 15–25 per o. i. f.; rare cocci; culture, marked growth but not confluent. Lavage lumbar to cistern with 15 cc. saline followed by 20 cc. antipneumococcus serum. Cistern needle withdrawn and left frontal trephine done. Injected very slowly 6 cc. serum. In view of the few organisms in the smear, it was felt that a second frontal trephine was not necessary.

45 hours—temperature 103.6°; unchanged clinically. No treatment.

66 hours—temperature 103.7°, clinically unchanged.

70 hours—diagnostic lumbar puncture; cells 3–5 per o. i. f.; cocci numerous.

72 hours—cistern and lumbar punctures; lavage of 15 cc saline lumbar to cistern; followed by 15 cc. serum. First cisternal smear shows several hundred pneumococci per field; smear following treatment shows diminution and agglutination of cocci. Animal left head down for 30 minutes

90 hours—temperature 102.7°, irritable but otherwise not notably worse. Cistern and lumbar tap. Cisternal fluid, yellow, purulent; contains about 150 cells per field; cocci markedly decreased and phagocyted. Culture: confluent growth. Lavage lumbar to cistern with 15 cc. serum. Rapid ether recovery.

114 hours—temperature 100.9°; weakness of hind legs; irritable; cistern and lumbar punctures; fluid nearly clear. Second trephine done.

Smears: lumbar—rare cells; no organisms seen. Cistern—rare cells; rare agglutinated pneumococci.

Cultures: fair growth.

Injected 2 cc. serum *via* each frontal trephine. 6 cc. lumbar subarachnoid space. 4 cc. cisterna.

Since sterilization was incomplete after several tıeatments, it was decided to test out the efficacy of ethylhydrocupreine hydrochloride (optochin) intrathecally. This effect is apparent in the continued protocol.

138 hours—temperature 99.4°. Unable to stand; lethargic. Lumbar and cistern punctures under ether. Cisternal smear: cells 5–10 per o. i. f.; cocci 15–20. Lavage with 15 cc., 0.0002 per cent ethylhydrocupreine hydrochloride in saline. Normal recovery.

168 hours—temperature 102°. Lumbar and cistern punctures under ether; cisternal fluid purulent; cocci very numerous. Injected 6 cc. 0.02 per cent optochin.

186 hours—usual double puncture; fluid contains hundreds of pus cells per field; cocci are diminished and markedly phagocyted. Injected 8 cc. of mixture of 15 cc. 0.2 per cent optochin and 5 cc. serum. Breathing ceased but was immediately renewed upon giving artificial respiration. Culture: rare colonies.

210 hours—dead. Autopsy· Moderate exudate over cord; brain congested; easily broken, fibrinous adhesions about cistern and over convexity; moderate seropurulent exudate at base and in both lateral ventricles; the latter are considerably dilated (Fig. 4). A small puncture wound in the floor of the fourth ventricle, just above calamus, with small hemorrhage extending into the central canal of the cord; this was the probable cause of death and is the inevitable result of frequent successive punctures into a dangerous region where working space is small. Smears from all regions show pneumococci—scanty in the cord, moderate in cistern and over convexity, fairly abundant at base and in lateral ventricles. The cellular reaction parallels the pneumococcus distribution in intensity. The principal feature in all smears is the extraordinary amount of phagocytosis and destruction of pneumococci. Many cells are loaded with bacterial débris (Fig. 5). Cultures: all regions positive; heart blood: no growth.

Summary: A mild initial infection; first treatment incomplete; considerable delay in instituting subsequent treatments; the latter usually not complete. Finally recourse to optochin in low concentration, resulting in exacerbation in growth of organisms. Gradual tendency toward sterilization with higher drug concentrations; course interrupted by traumatic death. A total of seven treatments, usually only partial in distribution.

As will be seen from the protocol, the initial growth of organisms in the spinal fluid was described as mild. We have made certain observations as to the rate of increase of these mild initial growths; for example, the fluid in one dog infected with 4½ million pneumococci is described as follows: 19 hours, 7 diplococci found in the entire smear; 23 hours, an average of 10 per oil immersion field; 42 hours, upwards of 100 cocci per field. The speed of progress of the infection becomes very evident and the significance of even a delay of a few hours in instituting treatment is obvious.

One dog afforded opportunity to compare the difference in numbers of pneumococci in washed and unwashed areas of cortex after a single large lavage, followed by optochin.

Dog 17.—Fox terrier, female; weight 5 kilos. March 30, 1927, 4.40 p.m., ether; cistern puncture; clear fluid. Injected 5,200,000 Type I pneumococci (6 hour dextrose broth culture; small transplant; growth 260 million per cc.). Good recovery.

24 hours—temperature 100.7°; in bad condition; irritable; unable to stand; convulsive. Morphine; ether; combined cistern and lumbar punctures; fluid cloudy; cells fairly numerous, hundreds of cocci per field. Lavaged from lumbar to cistern 50 cc. warm saline followed by 7 cc 0.2 per cent optochin. On the 7th cc., respiration ceased, pulse continued strong, but short period of artificial respiration failed to revive, a drug death. Autopsy showed the cord, cistern, and cerebellum covered with a moderate film of exudate; a small triangular area of cortex just above the cerebellum was similar in appearance, but the base and balance of the cortex, where no lavage had penetrated, were overlaid with thick yellow pus and myriads of cocci. There was moderate bacteremia. Figs. 6 and 7 are offered for comparison of smears from washed and unwashed cortical areas. We are, of course, assuming that the initial distribution of cocci was the same.

This animal died from the effect of the drug on the respiratory center, and the manner of death typifies that which we have regularly seen with overdoses of drug. Respiration suddenly becomes shallow and slow, but pulse continues strong until some time after breathing has ceased. This effect on the respiratory center is transitory, and if artificial respiration is vigorously applied, with, if necessary, the addition of intracardiac adrenalin, it is almost always possible to revive the dog. When large concentrations of drug were used, respiratory difficulties were common, but deaths were few; artificial respiration was in two instances maintained for 20 minutes, during which time the

pulse ceased, only to be renewed by adrenalin injections. Recovery ensued.

A word may be said about the drug concentration which has seemed safe. This has been found not to exceed that present in a mixture of 15 cc. of antiserum and 0.75 cc. of 1 per cent optochin. When no antiserum is used, this dose is too high; a safe maximum has not been determined for drug not diluted with serum. If much pus is present, an animal may withstand higher concentrations of drug than would otherwise be the case; if lavage is very effective and the return fluid practically clear, the drug concentration should never exceed that given above. Great caution should be observed if the fluid return through the cistern needle is blocked. With excellent drainage, the drug effect on the respiratory center appears a function of percentage concentration of optochin in the lavaging fluid, rather than one of total cc. of mixture lavaged through the meninges.

Having determined the dosages of optochin-serum mixture which were tolerated with little or no respiratory disturbance, systematic treatments were again undertaken. The results are evident from the following representative protocols. For convenience, we are illustrating the type of result obtained in mild initial infections and comparing these with that secured in severe initial infections.

Dog 25.—Female hound; weight 7½ kilos. April 25, 1927, 3.45 p m., ether; cistern puncture; clear fluid. Injected 10,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 800 million per cc). Good recovery.

19 hours—temperature 103.4°. Sluggish; has eaten. Morphine; ether; lumbar and cistern punctures; fluid cloudy; contains 15–20 cells per o. i. f., and about 1 diplococcus to every 3 fields; lavage attempted, but fluid came through with great difficulty and was blood-tinged. Lavage consequen'ly abandoned. After drainage a mixture of serum 10 cc. and 1 per cent optochin 0.5 cc. was distributed equally between cord and cistern. Double frontal trephines were done and 3 cc. of a similar mixture introduced on each side. Cistern culture, abundant growth.

42 hours—temperature 101.3°. Sluggish; lateral nystagmus (traumatic?). Morphine; ether; double puncture; yellow opalescent fluid; cells 15 per o. i. f.; only 1 coccus seen; culture grew but 2 colonies. Injected 10 cc. saline with poor recovery; followed by 5 cc. optochin-serum mixture in lumbar subarachnoid space, and 2 cc. in each frontal. Good recovery.

66 hours—temperature 99°. No treatment. Subsequent uneventful recovery.

Dog 26.—Female hound; weight 5½ kilos; a companion dog to the preceding. Infected with 8,000,000 pneumococci from the same culture.

20 hours—temperature 102 9°. Sluggish. Morphine; ether; quadruple puncture. Cisternal fluid cloudy; cells 15–30 per o. i. f.; cocci average 1 to every 2 fields. Saline lavage lumbar to cistern successful at first, but return then failed; repeated washing and draining *via* cisternal needle. Injection of mixture 15 cc. serum and 1.5 cc. 1 per cent optochin, divided 5 cc. cord, 5 cc. cistern, 2 cc. each frontal. Culture, abundant growth.

42 hours—temperature 102°. Clinically normal, usual double puncture, lumbar and cistern. Fluid clear; contains 2–3 cells per field, about half small lymphocytes. Introduced 6 cc. serum into lumbar subarachnoid space and placed head downward No drug; no frontal treatment. Culture sterile.

66 hours—temperature 101° No treatment.

Remained unchanged until the 16th day Found prostrate and convulsive; snapped and salivated and was thought to have rabies Chloroformed. Typical pneumococcus meningitis, generalized over convexity and base; cord relatively free. Smears show large numbers of pus cells and diplococci, fairly good phagocytosis. Ventricles dilated; purulent ventriculitis Type I pneumococci recovered from all regions including even the ethmoid cells

Summary: Mild initial process; sterilization incomplete; reinfection delayed and from some focus so small that in the period between the first treatment and the cistern tap, 24 hours later, no reinfection of the cisternal fluid had occurred, thereby giving a false negative culture.

The subsequent protocol is illustrative of the importance of residual foci in reinfecting meninges not completely sterilized. Experiments such as this are the type which convince us of the inefficacy of partial lavages as practiced by Kolmer.

Dog 20.—Female airedale; weight 9½ kilos. Has canine distemper April 6, 1927, morphine; ether; cisternal puncture; clear fluid; injected 10,500,000 Type I pneumococci (6 hour dextrose broth culture; growth 1,200,000,000 per cc.). Good recovery.

19 hours—temperature 102 8°; lethargic, morphine; ether; lumbar and cistern punctures; fluids purulent, contain several hundreds of cells and hundreds of pneumococci per o i f (Fig 8) Essentially no phagocytosis; a very severe infection. Lavage lumbar to cistern with 30 cc. saline followed by 11 cc. of mixture of serum 10 cc. and 1 per cent optochin 0.5 cc. Double frontal trephine with injection of 3 cc. of similar mixture on each side; 10 cc. of 800 unit antibody solution (Felton) intravenously; spinal fluid culture, confluent growth.

24 hours—temperature 100°. Ether; double puncture; lumbar and cistern; fluid clearer, cells 50 per o. i f; some contain phagocyted pneumococci (Fig. 9); practically no free organisms; culture, scattered colonies. Lavage lumber to cistern with 10 cc. saline followed by 0 035 per cent optochin-serum 7 cc.; no frontal treatment.

43 hours—temperature 98.8°; sluggish but otherwise unchanged; morphine; ether; lumbar and cistern punctures; fluid yellowish, opalescent; cells 10–35 per o. i. f.; no organisms seen. Injected 6 cc. serum, lumbar subarachnoid space and placed head down. 75 cc. intraperitoneal saline. Culture yielded about 300 colonies from a 0.5 cc. planting.

65 hours—temperature 102.7°; quite ill; nose a mass of thick pus; cistern and lumbar punctures; fluid a culture of pneumococci, hundreds per field (Fig. 10); cells rare; no agglutination nor phagocytosis. Represents a massive reinfection of a nearly sterile region, following cessation of complete treatments. Injected 8 cc. 0.05 per cent optochin-serum mixture divided between cord and cistern and 3 cc. into each frontal.

90 hours—temperature 99.6°; weaker; able to stand; ataxic; no change in distemper; ether; cistern and lumbar punctures; no fluid obtained. Gave 5 cc. lumbar, 1 cc. cistern, 3 cc. each frontal of 0 1 per cent optochin-serum. Intraperitoneally 150 cc. 10 per cent glucose; milk by stomach tube.

114 hours—temperature below 94°; chloroformed. Autopsy: Material suggesting chicken fat clot in fourth ventricle, extending down over cervical cord. Explains the last "dry tap." Fibrin at base, about pituitary and optic chiasm. Cord elsewhere relatively clear; anterior convexity relatively clear; meninges over posterior convexity opaque; exudate purulent; some pus in lateral ventricles.

Region	Cells per o i f	Cocci	Culture
Cord (lumbar)	5–10	Rare phagocyted; very rare free	0
Cistern	20–75	Moderate; free and phagocyted	+
Anterior convexity	5–25	Only 2 cocci in entire smear	0
Posterior " (Fig. 11)	75	Rare, phagocyted	+
Base	25	Moderate; free and phagocyted	+
Lateral ventricle (Fig. 12)	75	Moderate, phagocyted; more, free	+

A pneumonia practically lobar in distribution, entire left lung; culture, *bronchisepticus*. Negative heart's blood.

Summary: Initial massive infection; tendency to sterilization with treatment; "reinfection" with cessation of treatment; renewed tendency toward sterilization with resumption of treatment. Main focus of infection lateral ventricle; course of disease interrupted by fatal outcome of distemper.

As examples of severe infections sterilized slowly and progressively by successive complete treatments in one instance, seven in all, necessitating altogether ten etherizations, partially for treatment and partially for diagnosis, the following may be offered.

Dog 29.—Male hound; weight 7½ kilos. May 2, 1927, 4.00 p.m., morphine; ether; cistern puncture. Clear fluid. Injected 10,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 680 million per cc.). Good recovery.

24 hours—temperature 102.2°. Sluggish; lumbar puncture; fluid under increased tension, opalescent, yellowish; cells 50 per o. i. f.; diplococci 5–12 per field. Not treated. Culture, confluent growth.

42 hours—temperature 103.7°. Sluggish but able to stand Morphine; ether; quadruple puncture; lumbar fluid yellowish, opalescent; cells 8–10 per o. i. f; diplococci about 100 per field; similar cisternal fluid (Fig. 13). Lavage attempted, but proceeded with difficulty and with poor return through cisternal needle; abandoned. Injected 7 cc. of mixture of serum 10 cc., optochin 1 per cent, 0.5 cc. cisterna, 3 cc. lumbar, and 2 cc. similar mixture in each frontal subarachnoid region Cultures, confluent growth.

66 hours—temperature 102.9°. Clinically unchanged. Morphine; ether; quadruple puncture. Cisternal fluid clearing; cells rare; diplococci less than 20 per field. Lavaged lumbar to cistern with 10 cc of saline; injected serum-optochin mixture as above. Gave 100 cc. intraperitoneal glucose. Culture, confluent growth

90 hours—temperature 102.8°. Unchanged. Quadruple puncture; fluid more cloudy; cells 20–40 per field; cocci double in number. Last treatment repeated. No lavage Culture, confluent growth.

96 hours—treatment repeated with exception of frontal injections. Cisternal fluid cloudier; cells increased but what few pneumococci are present are all phagocyted. Culture, few colonies.

114 hours—temperature 102.6°. Clinically unchanged. Morphine; ether; cisternal puncture; fluid scanty; 6–7 diplococci per field; culture, colonies increased. Complete treatment as above with exception of left frontal; no lavage.

138 hours—temperature 104°; unchanged; has eaten and drunk; stands and wags tail. Ether; quadruple puncture; lavage lumbar to cistern with 15 cc. serum-optochin mixture (serum 15 cc, optochin 0.75 cc. of 1 per cent solution); 1.5 cc. same mixture, both frontal trephines. Fluid clearing; cells 5–10 per field; no cocci seen. Culture, scanty growth.

162 hours—temperature 103.7°; canine distemper; less active; will not stand without assistance Ether; lumbar and cistern punctures; cells 4-5 per field; no cocci; *culture, sterile;* lavage with 14 cc. serum-optochin mixture as above. Intraperitoneal glucose, 100 cc.

186 hours—temperature 102.7°; distemper worse; thick pus flowing from nostrils. No treatment.

210 hours—unchanged; diagnostic cistern puncture; rare cells; no cocci; injected 5 cc. of serum as prophylactic; culture, negative.

288 hours—very ill from distemper. Subnormal temperature. Chloroformed. Bilateral bronchopneumonia (*B. bronchisepticus*). Entire brain and cord grossly normal save for slight excess of small lymphocytes and endothelial leucocytes in

cisternal and ventricular fluids. Cultures taken from all regions, including the entire fluid contents of the lateral ventricles, failed to give growth.

Microscopically some slight generalized infiltration of the meninges by endothelial leucocytes, many fatty, a few with blood pigment; a typical late clearing up stage In addition, collections of lymphocytes in the region just beneath the ependyma of ventricles. An organizing (purpuric?) hemorrhage of gray matter of cord.

Dog 32.—Female hound, weight 5 kilos May 4, 1927, 4 05 p m., morphine; ether, cistern puncture; clear fluid. Injected 7,500,000 Type I pneumococci (6 hour dextrose broth culture; growth 750 million per cc.). Good recovery.

22 hours—temperature 104°; sluggish; irritable. Ether; lumbar puncture; fluid seropurulent; cells 15–20 per o i f , cocci upwards of 150 per field (Fig. 14); cisternal fluid opalescent; cells rare, cocci 20–30 per field. Lavage, lumbar to cistern with 15 cc. saline. Frontal trephines, injection of mixture of 15 cc. antiserum and 0 75 cc. 1 per cent optochin as follows. lumbar subarachnoid space, 7 cc , cisterna magna, 3 cc., each frontal trephine, 2 cc. Culture, confluent growth.

42 hours—temperature 102 4°, stands weakly, attempts to walk; drinks Ether; quadruple puncture, cisternal fluid clearing, cells 20–30 per field; no cocci seen; culture, colonies numerous but growth not confluent, injected serum-optochin mixture (as above) 7 cc. lumbar, 3 cc. cistern, 1 cc each frontal

66 hours—temperature 101°; marked loss of weight; weakness hind legs Ether; quadruple puncture; fluid almost clear; cells 2–3 per field and cocci about 1 in every 2 or 3 fields; culture, colonies increased. Treatment as at 42 hours. Intraperitoneal glucose.

90 hours—temperature 98°; definite partial paraplegia hind legs; probably traumatic. No treatment

114 hours—temperature 98 6°, unchanged; diagnostic cistern puncture; abundant crystal clear fluid; lymphocytes less than 1 per field; no organisms. Plated 2 cc. of fluid; no growth.

138 hours—temperature 99.3°; diagnostic puncture; negative culture. Subsequent course uneventful, persistent partial paraplegia.

The following may be offered as an example of a case where two complete treatments failed to sterilize the meninges appreciably. The condition was complicated by distemper.

Dog 34.—Young female collie pup. May 10, 1927, 4 00 p m., ether; cisternal puncture; clear fluid. Injected 9,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 610 million per cc.). Good recovery.

19 hours—temperature 104°; sluggish, has developed severe distemper.

23 hours—ether; quadruple puncture, cistern and lumbar fluids seropurulent; cells 50–75 per field; diplococci very numerous. Lavage lumbar to cistern with

20 cc. saline, followed by lavage with serum-optochin mixture (serum 15 cc , 1 per cent optochin 0.75 cc.), lumbar to cistern 15 cc.; frontal trephines 2 cc. each of mixture of similar proportions. Intraperitoneal glucose, 100 cc. Culture, confluent growth.

42 hours—temperature 101.5°, unchanged. Quadruple puncture; complete treatment as above; 100 cc. glucose intraperitoneally. Smear. an 80 per cent reduction in organisms; cells average 12 per field Culture, confluent growth.

66 hours—temperature below 94° Chloroformed In gross the brain and cord show only a trace of exudate, microscopically smears contain relatively few cells; diplococci present in large numbers and save in the cord region, where there is some phagocytosis, no evidence is seen of beneficial effect of treatment. Marked purulent bronchitis, but death is undoubtedly meningeal Cultures all give confluent growth; heart's blood, rare colonies. Histologically severe, diffuse leptomeningitis.

Comments.

Since optochin is somewhat under suspicion in view of its apparent tendency to produce transient amblyopia, we have endeavored to detect visual disturbances in recovered dogs. So far as was ascertained, none occurred; no ophthalmoscopic examinations were made. Some of the recovered dogs are deaf, but we know that in meningococcal meningitis permanent deafness may result.

A word may be said about lateral ventricular punctures. This has not been done in the dog; it is fairly certain that sterilization would be hastened and that more recoveries would have resulted had this been a routine procedure. Whereas the lateral ventricle of a dog, in the region where puncture is desirable, is a mere slit unless pathologic dilatation has occurred, it should offer no great problem in man. It is apparently the most difficult region to reach with serum by the methods applied in this study. In this respect the base in the region of the chiasm shares with the lateral ventricle and in case these methods should ever be employed in treating human disease, failure to sterilize by the quadruple puncture route should make one think seriously of ventricular punctures and punctures through to the base. The important thing in securing a cure is contact of all regions with the optochin-serum mixture—contact complete and frequently repeated. Undoubtedly lavage is a valuable adjuvant, but lavage alone cannot rid the meninges of organisms and if even a very small number remains, our experience shows that severe reinfection is almost inevitable. The object of treatment should be to wash out as many

organisms as possible and to control the residual bacteria by a pneumococcidal drug and a phagocytosis-producing antiserum.

One of the most hopeful and surprising things observed in the entire study was the manner in which lavage may be accomplished even in the presence of a massive exudate, it being possible to lavage a cord in an animal having almost frankly purulent cisternal and lumbar fluids, until the return fluid is nearly crystal clear; full cognizance is, however, taken of the fact that no matter how long one washes, within reason, cells and pneumococci blocked in fibrin meshes, the former certainly in large numbers, will remain. It has seemed that it is easier to lavage out organisms than it is cells, since the latter are larger and the network of fibrin offers more resistance to their free passage. As judged by the pictures of phagocytosis in foci fairly distant from the site of injection, the immune serum penetrates the exudate very satisfactorily.

No statistics of cures are presented. In view of the prevalence of traumatic deaths after frequently repeated cistern punctures, drug deaths, when high optochin concentrations were used, and especially mortality from epidemic canine distemper, such statistics would be meaningless. The fact that progressive sterilization of the meninges by methods employed in this study is possible has been established in our opinion without question.

Pathology.

The microscopic pathology has been studied in all animals dying of the disease, and in certain recovered dogs which succumbed to intercurrent disease (distemper) after becoming sterile. Sections show a rapidly spreading, fibrinopurulent leptomeningitis, which as early as 23 hours after infection may involve all regions of the meninges (Fig. 15). In a control dog, or in a treated animal in which the disease was not checked, the exudate reaches large proportions; the cord dura is invaded; the spinal nerve roots are involved (Fig. 16); there is an inflammatory process in the epidural fat (Fig. 17). Invasion and destruction of the choroid plexuses, superficial encephalitis, spread *via* the Virchow-Robin spaces are common (Figs. 18–20). An empyema of the third or fourth ventricle with extension into the central canal of the cord may occur (Fig. 21). Probably, too, the latter may

be reached from the ventral fissure. Once in the central canal the process may destroy the ependyma and invade the region of the commissures, and from thence the cornua by following a perivascular route, giving rise to a myelitis. Invasion, at least to any great extent, of the lateral ventricles occurs relatively late, probably because of the direction of flow of the spinal fluid; in other words, the lateral ventricles lavage themselves. With the development of a blocking exudate over the convexity or in the fourth ventricle or aqueduct, empyemas with dilatation of the lateral ventricles inevitably result. With the subsiding of the process, polymorphonuclears disappear and are replaced by fatty and phagocytic endothelial leucocytes and small lymphocytes, which cells apparently may persist for some time. Such was the picture observed in Dog 29, Fig. 22 (see protocol). Slight subependymal and peripheral cortical gliosis may be found.

Reinfection Experiments.

Five recovered dogs, together with a control, were reinfected to see if any immunity to meningeal infection existed. These animals (Dogs 1–5) were reinoculated 103, 79, 63, 47, and 42 days respectively after primary infection. Four ran an atypical course. The first two developed a very high grade, early leucocytosis; in one of these no organisms were observed in the 24 hour spinal fluid smear, and a culture gave no growth. Dog 1 died of severe purulent meningitis in 114 hours. Dog 2, whose culture was sterile 24 hours after infection, showed organisms at 66 hours; the number of cocci in the smears increased very slowly and at 7 days they were no more numerous than frequently seen after the 1st day in a primarily infected animal. As organisms increased, leucocytes diminished; phagocytosis was rarely seen and can scarcely account for the slow development of the disease. In none of the dogs were agglutinins demonstrable in the spinal fluids. Dog 2 died the 9th day. Dogs 3 and 5 gave negative cultures, and smears contained but 20–30 leucocytes per field 24 hours after infection; by 66 hours fluids were crystal clear and sterile. Dog 4 ran a typical course, but was sterilized by two complete optochin-serum treatments. This sterilization was unusually rapid. The control dog died typically in 48 hours. Hence a degree of increased resistance remained within the meninges.

RÉSUMÉ AND CONCLUSIONS.

1. Extensive acute, fibrinopurulent meningitis may be produced in dogs by the intracisternal injection of virulent Type I pneumococci.

2. Given an equal number of virulent infecting organisms, the rate of establishment of infection depends upon the phase of growth and the quantitative growth per cc. of culture plus an uncontrollable individual factor in the animal.

3. The pathology of pneumococcal meningitis is discussed. It resembles very closely the similar disease in man.

4. Systematic lavage and treatment with optochin-serum mixtures by the method of quadruple puncture, as described above, have resulted in cures of Type I pneumococcal meningitis in dogs.

5. The important factor in obtaining cures is to bring all regions of the meninges into frequent contact with the therapeutic agent.

6. In the absence of such contact, incomplete sterilization results and "reinfection" is almost inevitable.

7. Protocols show the necessity of repeated negative cisternal fluids, both on smear and on culture, before sterilization can be assured.

8. Recovered dogs subjected to meningeal reinfection show some degree of resistance.

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EXPLANATION OF PLATES.

Plate 16.

Fig 1. Control Dog 3. Massive basal exudate.

Fig 2 Control Dog 7. Smear from convexity. × 1000.

Fig. 3. Distribution of dye introduced *via* left frontal trephine

Plate 17.

Fig. 4. Treated Dog 10 Dilated ventricles; purulent ventriculitis.

Fig. 5. Treated Dog 10. Ventricular smear showing extensive phagocytosis after ethylhydrocupreine hydrochloride treatment. × 1000.

Fig. 6. Treated Dog 17. Smear from posterior (lavaged) cortex. × 1000.

Plate 18.

Fig. 7. Treated Dog 17. Smear from anterior cortex unaffected by lavage and chemo-serotherapy. × 1000.

Fig. 8. Treated Dog 20. Initial 19 hour cisternal smear. × 1000.

Fig. 9. Same dog. 24 hour smear (after one treatment). × 1000.

Fig. 10. Same dog. 65 hour smear, showing result of cessation of treatment. × 1000.

Plate 19.

Fig. 11. Same dog. Smear from posterior convexity taken at 114 hours. Rare phagocyted cocci. × 1000.

Fig. 12. Same dog. Lateral ventricle smear. × 1000. Lateral ventricle a residual focus of infection.

Fig. 13. Recovered Dog 29. Cisternal smear before treatment. × 1000.

Fig. 14. Recovered Dog 32. Cisternal smear before treatment. × 1000.

Plate 20.

Fig. 15. Control dog, showing extent of exudate at 23 hours. × 50.

Fig. 16. Control dog. Massive exudate. Invasion of spinal nerve. × 50.

Fig. 17. Treated dog. Accidental death. Inflammatory exudate in epidural fat. × 50.

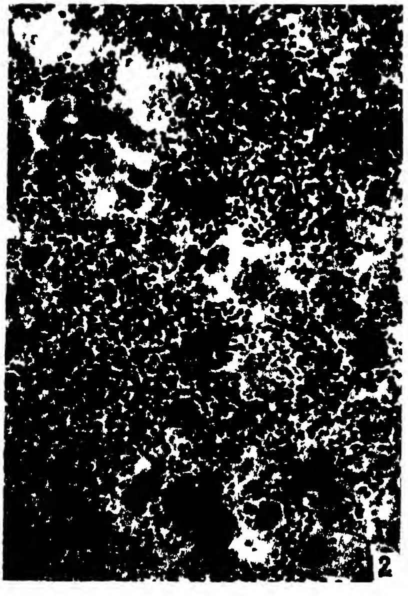
Fig 18. Control dog. Purulent ventriculitis; superficial encephalitis; destruction of choroid plexus of lateral ventricle. × 50.

Plate 21.

Figs. 19 and 20 Control dog. Perivascular infiltration of cortex. × 50.

Fig. 21. Control dog. Empyema of central canal of cord; myelitis. × 50.

Fig. 22. Treated Dog 29. Sterile cultures; death from distemper; meninges essentially negative. × 50.



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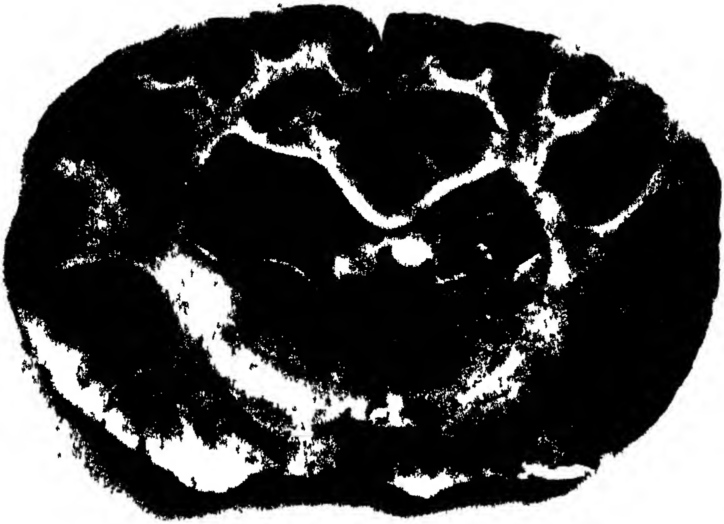

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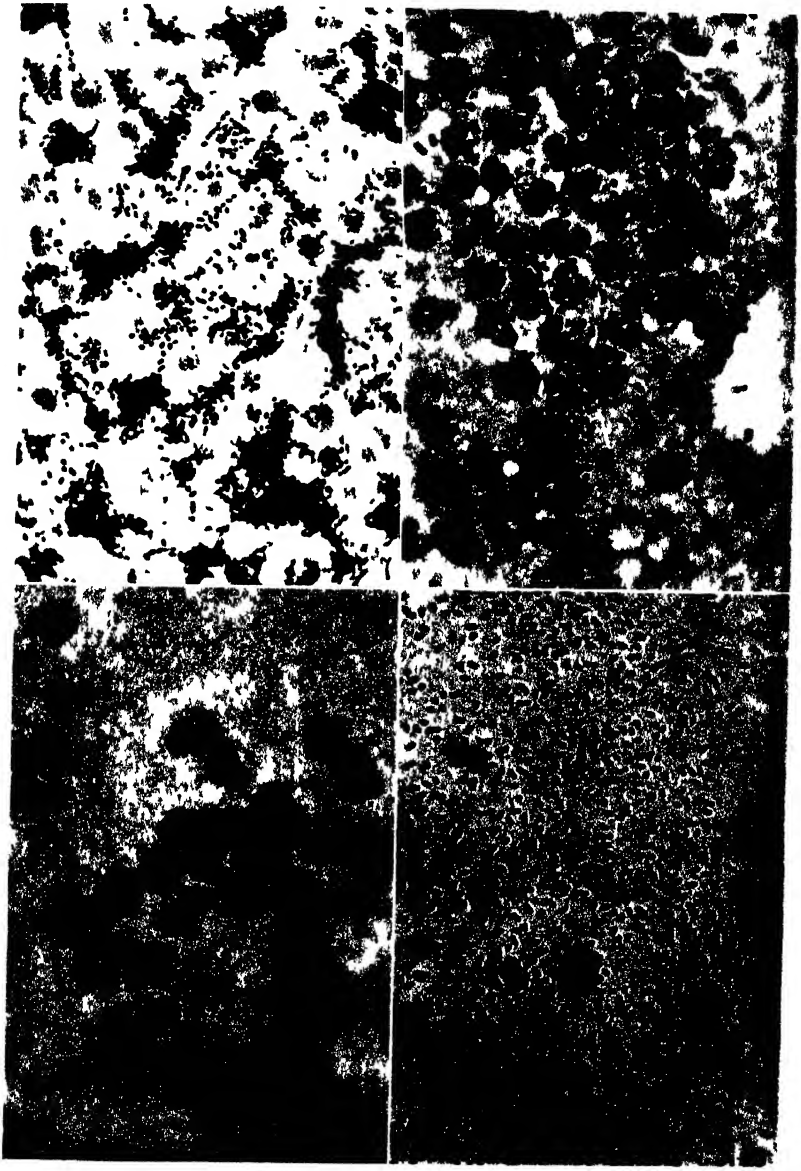

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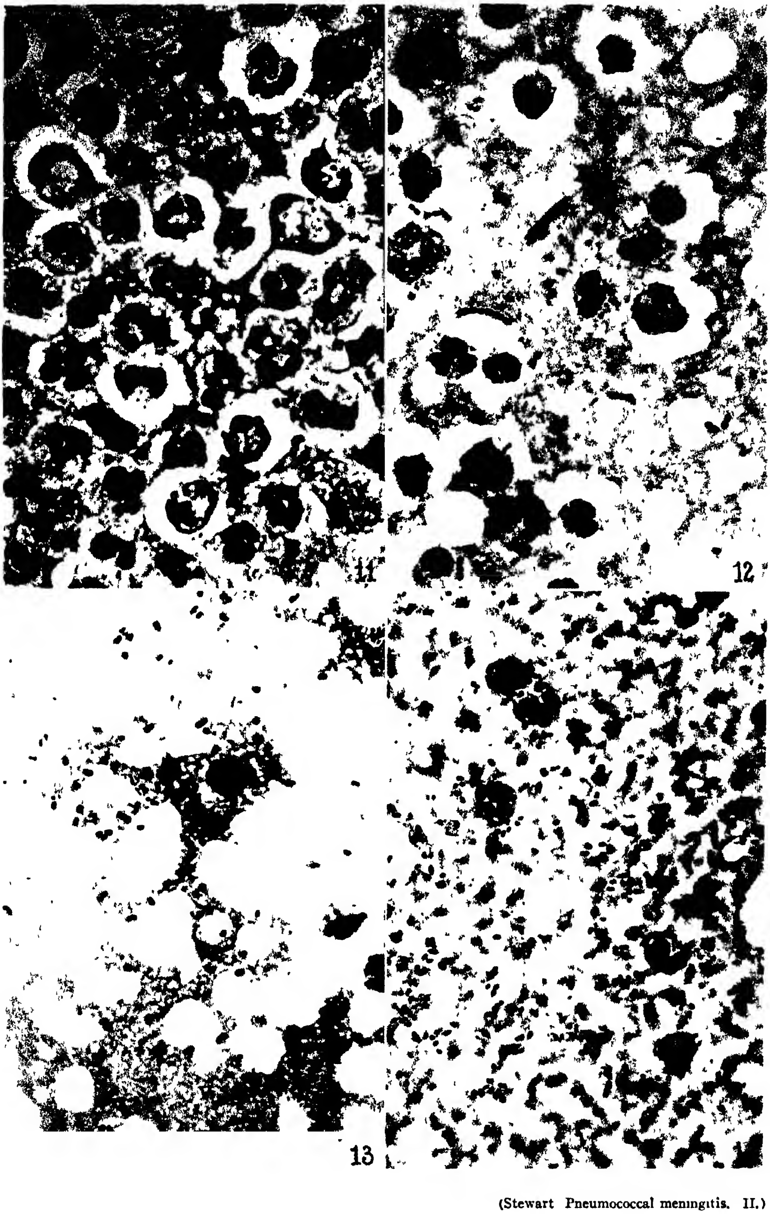

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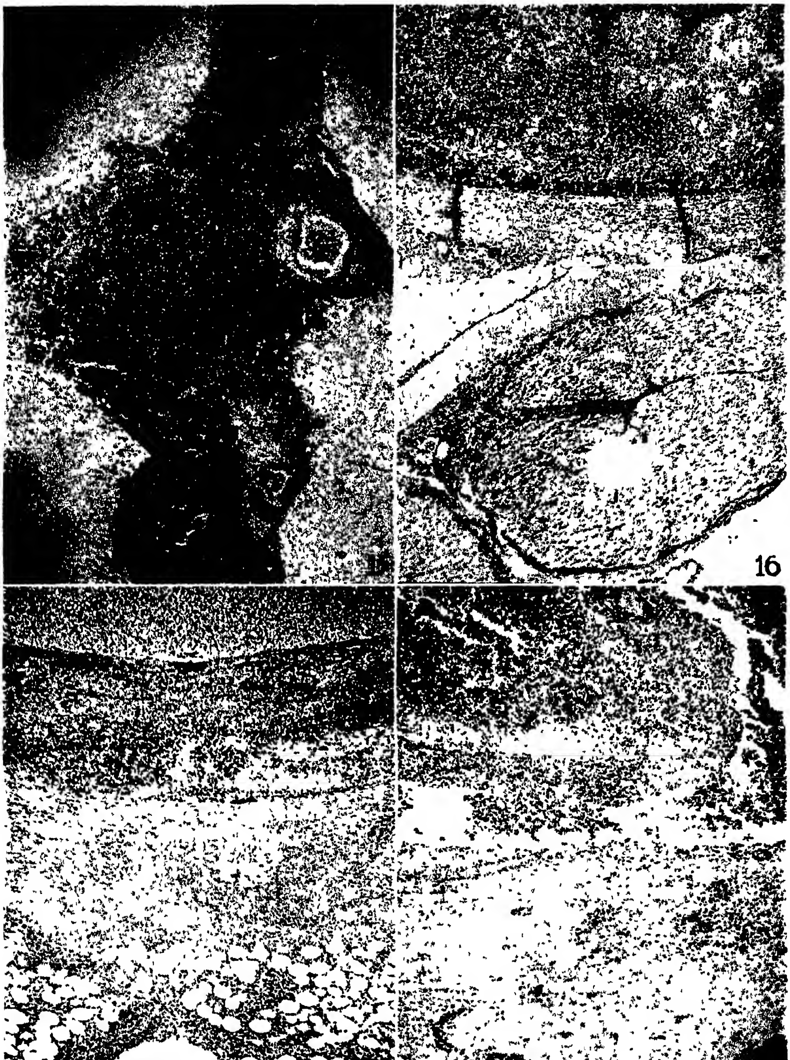
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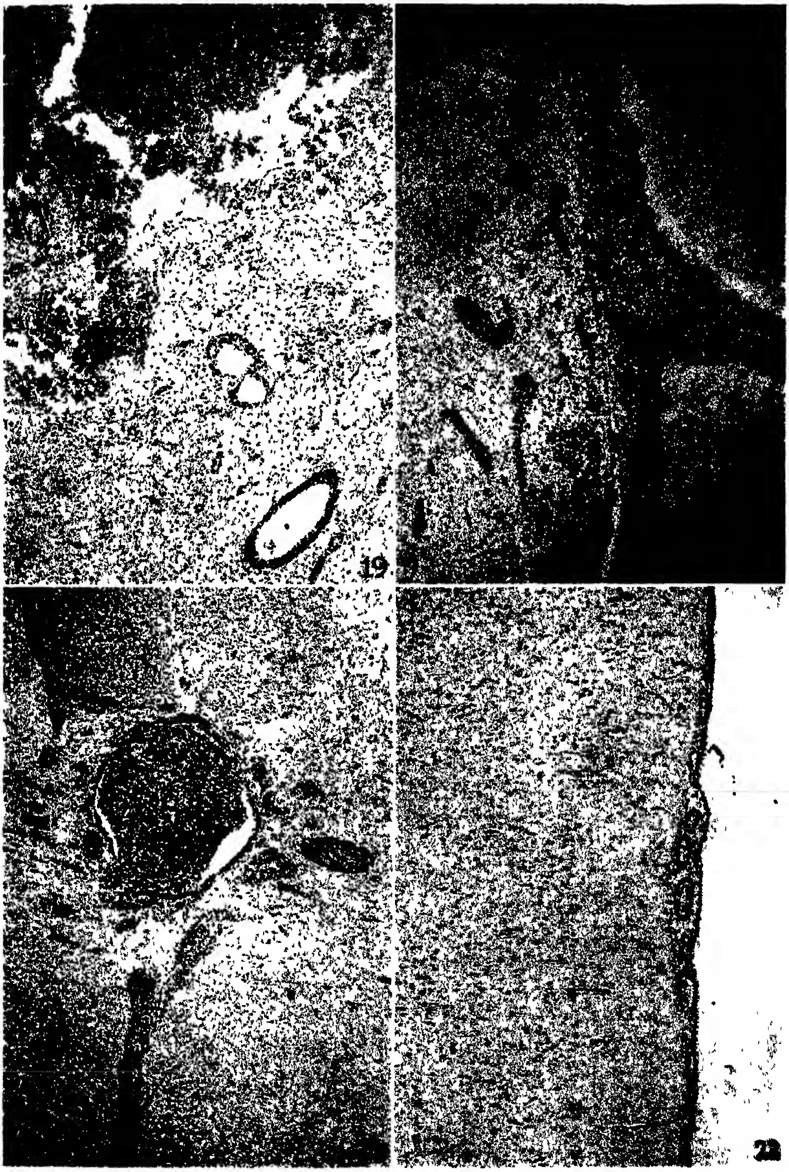

Stewart Pneumococcal meningitis II)

[Reprinted from THE AMERICAN JOURNAL OF ROENTGENOLOGY AND RADIUM THERAPY, June, 1927, Vol. xvii, No. 6, pp. 667–670.]

APPARATUS FOR MAKING ROENTGEN-RAY DOSAGE MEASUREMENTS.

BY HARRY CLARK, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

The rate of ionization of air in a properly designed and calibrated chamber is generally considered to be the best index that we have of the dosage of roentgen rays used for therapy. The habit of making such measurements has not spread very rapidly, however, one of the reasons being, presumably, that no standard equipment is available which is accurate and reliable, and at the same time simple, compact, and conveniently portable.

When the target distance is great or when it is desired simply to check the constancy of output of a tube, a large ionization chamber may be used and the current may be measured by means of a very sensitive galvanometer. On the other hand, one must use a small chamber to measure the rate of ionization at a point, approximately, and to integrate the effects of direct and secondary radiation. This means a small current and the use of an electroscope or an electrometer. Ideally, the chamber would be connected with the electroscope by means of a flexible conductor so that it could be moved and adjusted to any desired position without the necessity of moving, readjusting, and checking the electroscope. How to construct a properly insulated and protected flexible conductor is an unsolved problem, unfortunately.

It occurred to me some time ago that if the chamber were connected permanently to a small condenser in such a manner as to make a simple compact unit, this unit might be disconnected from the electroscope for the duration of the exposure to the rays, and returned to it afterwards for a measurement of the amount of charge lost. It would be possible, thus, to do away with the flexible conductor entirely. The single obvious disadvantage is that one would have to estimate the length of exposure of the chamber in advance; it will appear further on that this need give little trouble in practice.

Apparatus of this kind has been made and used, and it has been found to work very nicely. The particular instrument which I shall describe was designed to measure the relatively soft rays produced at from 30 to 100 kv. peak. It represents, of course, merely a first attempt to solve the problem; several changes will be made when it is reconstructed.

DESCRIPTION OF THE INSTRUMENT

Figure 1 shows the apparatus completely assembled; in Figure 2 some of the parts have been removed in order to show the essential features of the internal construction; *a* is the chamber unit; the rest will be called the electroscope.

The Electroscope. The insulated element of this unit comprises the gold leaf and its support, *b*, the upper set of plates, *c*, of a variable condenser, and the device, *d*, by means of which a connection may be established with the inner electrode of the chamber unit, it is mounted on a single amber insulator, *e*, so shaped as to insure that the apparatus can be taken apart and reassembled without changing the capacity of the condenser, *d*, is merely a pin, the head of which is supported on a light helical spring. The sensitiveness of the gold leaf may be adjusted by means of the screws, *f*. By turning the button, *g*, one connects the insulated element with a charging battery—7 45-volt B-batteries. The lower set of plated, *h*, of the condenser, is connected with the case, it is fed up or down by means of a screw attached to the drum *i*. The capacity scale on the drum was drawn after a very careful experimental study of the condenser. The travel of the drum is limited at both ends by means of stops. The capacity change is about 67.5 cm.

The Chamber Unit. This unit, which consists of a small chamber and a condenser, is shown connected with the electroscope at *a* in Figure 1 and separately, at *a* in Figure 2. The condenser consists of a small brass cylinder supported axially inside the brass tube which constitutes the visible body of the unit. The inner cylinder is $1\frac{1}{2}''$ long and $\frac{3}{8}''$ in diameter; it is mounted on amber at either end. The outer tube is $2\frac{3}{4}''$ long, and $\frac{3}{4}''$ in external diameter; the diameter of the hole exceeds that of the inner cylinder by only $0.01''$.

The ionization chamber is of pure graphite. The outer shell is

about 0.01" in thickness, the inner diameter being $\frac{3}{16}$", the inner electrode is a graphite rod $\frac{1}{16}$" in diameter The amber extends to the middle of that part of the graphite shell which is visible in the illustrations, the air space in the chamber is thus only about $\frac{5}{8}$" long

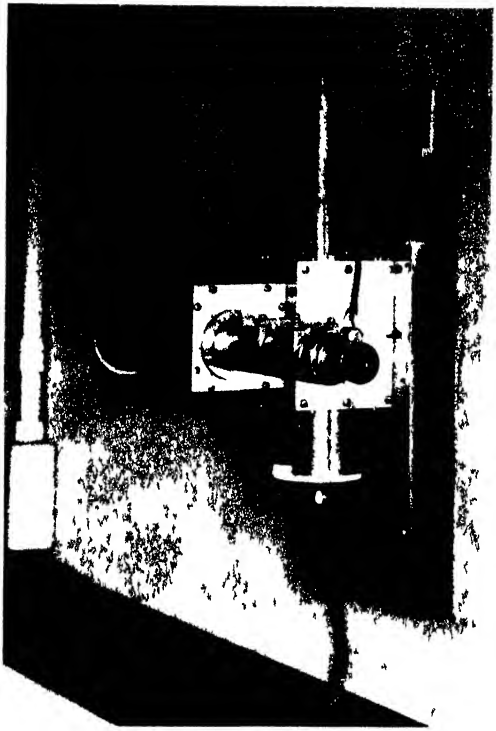

FIG 1 The apparatus completely assembled

The end of the condenser opposite to the chamber may be closed by means of the screw, *j*, the length of which is such that it very nearly makes contact with the inner cylinder In this end of the inner cylinder, there is a small conical depression, carefully centered, it serves to center the needle, *d*, when the chamber unit is being connected to the electroscope. A stop is provided in the tube, *k*, of the electroscope to limit the depth to which the chamber unit may be inserted.

The connection is made in exactly the same way every time, therefore, however carelessly the chamber unit may be inserted

The electrostatic capacity of the chamber unit is 70 cm

The volume of the air space in the condenser is about one-half as great as that in the chamber proper It is protected from the rays by

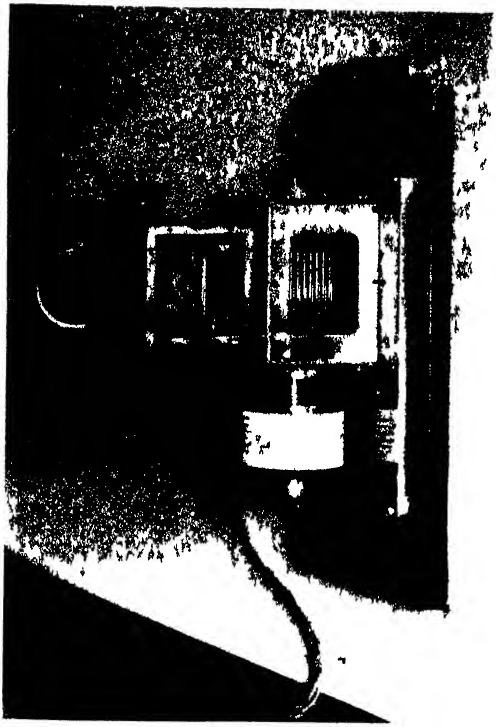

FIG 2 Showing the internal construction of the electroscope

$\frac{3}{16}$" of brass, however, so the leak is negligible for rays no harder than 70 kv When very hard rays are used the condenser must be protected with lead There is no good reason why the air space in the condenser may not be made much smaller, nor in fact any reason why the whole unit may not be reduced in size

No stress is put on the design of the chamber itself Beyond allowing

the amber to extend far enough into the graphite so that the brass would not cast shadows on the chamber at reasonable angles, it was attempted only to make the chamber of such shape that it could be saturated easily, and to make it light and thin enough to transmit very soft rays. The chamber could be improved without doubt; before rebuilding it, I intend to study the behavior of beryllium as a substitute for the graphite.

The range of usefulness of the instrument could be extended, of course, by providing several chamber units, each adapted to a particular range of intensity and wave length.

HOW MEASUREMENTS ARE MADE

With the chamber unit connected to the electroscope as shown in Figure 1, and with the drum, *i*, screwed nearly all of the way up, the insulated system is charged by turning the button, *g*, *on* and *off*. The leaf is then brought exactly to the middle point (zero) of the eyepiece scale by turning the drum one way or the other, and the capacity scale on the drum is read—the reading is called C_1. The chamber unit is now removed, closed with the screw, *j*, carried to wherever it is to be used, and exposed to the rays for a definite length of time. At any convenient time thereafter, the screw is removed and the chamber unit is connected again to the electroscope, after which the drum is unscrewed until the leaf comes to zero, and the scale reading, C_2, is recorded. The dose is given by $(C_2 - C_1)$ KD in which K is a constant, the value of which is 6.52 e-units per cm. or 1.056×10^{13} electrons per gram of air per cm., and D is the ratio of the density of air at 0°C. and standard pressure to the density at the time of exposure. D may be tabulated for various temperatures and pressures for quick reference. The measurement is to be taken as satisfactory provided $C_2 - C_1$ lies between 5 cm. and 55 cm. The dosage rate is found, of course, by dividing the total dose by the time of exposure.

The value of K depends on the value of V, the potential required to raise the gold leaf to zero, on the value of V, which is the "equivalent volume" of the ionization chamber, and on other quantities which have to do with the units in which the dose is to be expressed. V was measured with the aid of a potentiometer and a voltmeter when the electroscope was first set up and adjusted; it came out to be 313.6

volts. So long as the instrument is not readjusted it will remain constant. It should be noted carefully that v is not necessarily the same as the voltage of the charging battery.

v was determined by direct comparison of the chamber with another chamber, the behavior of which is well known. For unfiltered rays produced at 30 kv. peak, it is equal to 0.160 cc.; v varies to some extent with the hardness of the rays. If it be taken as constant and equal to 0.160 cc., the resulting error may be as great as 2.5 per cent for certain rays produced between 30 and 100 kv.

Preliminary experiments made it clear that 25 volts are enough to saturate the chamber current for the most intense rays with which I have to deal. This corresponds to a maximum value of 64 cm. for $c_2 - c_1$. To provide a proper margin of safety, I set the limit at 55 cm. So long, then, as $c_2 - c_1$ does not exceed 55 cm. it may be assumed safely that the current has been saturated throughout the exposure. Now the scale is divided to 0.1 cm. and the accuracy of the scale and the sensitivity of the leaf justify reading to about 0.02 cm. If then $c_2 - c_1$ is as great as 5 cm., it can be measured with fair accuracy. The length of the exposure may lie, then, anywhere between a certain minimum value and 10 or 11 times that value. Very little experience is required to enable an operator to estimate the exposure with reasonable certainty that the first trial will be satisfactory.

If the electroscope is properly cleaned, and mounted at some little distance from the roentgen-ray plant, no account need be taken of its natural leak; this is true even though the chamber unit remain disconnected from the electroscope for a considerable period—a day or two perhaps. If the apparatus is of massive construction, and if the gold leaf is so adjusted that it cannot be injured by careless handling of the variable condenser (as it is in my instrument) there is no reason why it should ever get out of order. If it is suspected that the chamber unit has been damaged by careless handling, its behavior can be checked in every detail without removing it from the electroscope. One particular advantage of this apparatus should not be overlooked; since the final adjustment of the leaf to zero is made by means of the variable condenser, the value of the battery voltage is of no great importance. If the batteries are set in paraffin, it should not be necessary to replace them for two or three years.

All things considered, the advantages of using apparatus of this kind for estimating dosage seem to me to be much more than sufficient to compensate for the single disadvantage—that the time of exposure must be estimated in advance. The apparatus is compact, fairly simple, and as nearly foolproof as anything of the kind can be made. It requires no attention over long periods. Once adjusted, its behavior may be checked up without the use of accessory apparatus of any kind. Aside from the matter of chamber design, which constitutes a problem quite independent of the nature of the rest of the apparatus, the measurements may be made as accurately as one wishes. Most important of all, the usefulness of the apparatus is not restricted to the immediate neighborhood of the electroscope.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1927, Vol. xi, No. 1, pp. 71–81.]

ON THE TITRATION OF BACTERIOPHAGE AND THE PARTICULATE HYPOTHESIS.

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(Accepted for publication, May 25, 1927.)

INTRODUCTION.

One of the methods most frequently used to estimate the concentration of bacteriophage in a fluid medium is that of serial dilution. If several parallel titrations of the same solution are made by this method, it will be found usually that the results are not entirely consistent; that, although in most cases the number of tubes in which the bacteria dissolve will be the same, let us say n, a few cases will yield $n \pm 1$.

Dr. Bronfenbrenner,[1] of The Rockefeller Institute, in whose laboratory many thousands of such titrations have been made on solutions of various degrees of concentration, estimates that, if the dilution factor be .1, about 85 per cent of such parallel runs yield the same value of n. This degree of consistency is about 40 per cent higher than one would expect if it is true, as is quite generally believed, that bacteriophage exists in the state of particles, a single one of which is sufficient when added to a culture of susceptible bacteria to start the destructive processes.

Dr. Bronfenbrenner's estimate is based largely on the general impressions gained by himself and his coworkers in the course of much experimental work rather than on definite records. The discrepancy between this estimate and the results of analysis is so great, however, that it deserves consideration. It should be checked by experiment. If the predictions of theory are upheld, it would constitute an interesting verification of the simple particulate hypothesis. If not, it would require a further consideration of the hypotheses on which the analysis

[1] I am indebted to Dr. Bronfenbrenner for his kindness in furnishing the material on which this paper is based

is based, which in itself might prove to be of interest. Inasmuch as the labor involved in making the experiments is very great, such a check can best be made as a by-product of titrations made for other purposes. A brief presentation of the analysis together with a discussion of the hypotheses on which it is based, may, therefore, be of interest.

The Serial Dilution Method.

The method will be explained briefly by an example We put 10 cc. of the solution to be titrated, which contains broth as well as bacteriophage, into the first of a series of test-tubes; into each of the other tubes, we put 9 cc. of sterile broth We now remove 1 cc. of the fluid from the first tube and introduce it into the second. After very thorough stirring, we remove 1 cc. from the second tube, using a clean pipette, and put it into the third tube We continue this process indefinitely, in so far as the theory is concerned The expectation of bacteriophage in any tube is, therefore, exactly one-tenth as great as that for the preceding tube. The quantity, 1, is called the dilution factor. Susceptible bacteria are now put into each of the tubes. In the first n tubes, they dissolve, in all of the others, they live and multiply. 10^{n-1} is taken as a measure of the concentration of the original solution

Statistical Treatment of the Problem.

It will be assumed for the present that the presence of one or more particles of bacteriophage in any tube always results in the dissolution of all of the bacteria, that particles neither dissociate nor coalesce during the process of dilution, and that none of the particles are lost by adsorption or otherwise. The effects of changing these hypotheses in various ways will be discussed later.

Let x = the exact number of particles of bacteriophage placed in the first tube,

p_n = the probability that the last (most dilute) tube in which the bacteria dissolve will be the nth tube of the series, and

a = the dilution factor.

In what follows, it will be assumed that $a = .1$ unless otherwise stated.

The probability that the $(n+1)$th tube receives *a particular one* of the particles originally in the first tube is a^n; the probability that it does *not* receive it is $1 - a^n$; and the probability that it receives ***none***

of the original x particles is, therefore, $(1-a^n)^x$. Likewise, the probability that the nth tube receives none is $(1-a^{n-1})^x$. These probabilities are not independent, however; whenever the nth tube receives none the $(n+1)$th tube *must* also receive none. In every other case in which the $(n+1)$th received none, the nth must have received *some*, and it must have *retained* them. Therefore, the probability, which is in effect p_n, that the nth retains at least one and the $(n+1)$th receives none is given by

$$p_n = (1 - a^n)^x - (1 - a^{n-1})^x \tag{1}$$

If x and n are infinite, xa^n being finite, this equation may be written

$$p_n = e^{-xa^n} - e^{-xa^{n-1}} \tag{2}$$

These are the fundamental equations with which we shall have to deal in what follows.

The Maximum Value of p_n for Small Values of n.

Let P_n = this maximum value of p_n, and
X_n = the value of x which corresponds to P_n.

If $n = 1$, it is obvious that $X_1 = 1$. Tube 1 must retain at least one particle, and the smaller the number of particles it receives, the less the probability that it will lose one of them to Tube 2. Equation (1) shows, then, that $P_1 = 1 - a$ which is .9.

If $n > 1$, we can find between what two consecutive integral values of x the desired value lies by treating x as a continuous variable. Accordingly, we set D_xP_n equal to zero. From equation (1), we find that

$$D_xP_n = (1 - a^n)^x \log_e (1 - a^n) - (1 - a^{n-1})^x \log_e (1 - a^{n-1}).$$

Setting this expression equal to zero, simplifying, and writing X_n in place of x, we have

$$X_n = \frac{\log[-\log(1 - a^{n-1})] - \log[-\log(1 - a^n)]}{\log(1 - a^n) - \log(1 - a^{n-1})} \tag{3}$$

in which the base of logarithms is arbitrary.

Column 2 of Table I contains the values of X_n found by setting a equal to .1, and n equal to 2, 3, and 4 in equation (3), and Column 3 contains the corresponding values of P_n found by substituting X_n in equation (1). These quantities cannot be less than the true values corresponding to the best integral values of X_n. Inspection of Column 3 shows that as n increases from 1 to 4, P_n apparently approaches a limiting value very rapidly. To make sure of this, we must find the value of P_n when n is infinite.

TABLE I.

1	2	3	4	5	6	7	8
$a = 1$						$a = .09$	
n	X_n	P_n	X'_n	p'_n	$\bar{p}_n$	P_n	p'
1	1 000	.900	7 27	.466		.910	.469
2	24 60	.706	76 6	463	604	.720	.467
3	255 0	698	770	.463	.602		
4	2558.	.697					
∞		.697		.463	.602	.717	.467

X_n is the value of x corresponding to P_n, the maximum value of p_n which in turn is the probability that the last (most dilute) tube in which bacteria dissolve is the nth tube of the series.

X'_n is the value of x for which $p_n = p_{n+1}$. At this point, p'_n, the degree of consistency of parallel runs, has a minimum value.

p_n is the mean value of p_n over the range of values within which p_n is greater than p with any other subscript.

The Value of P_n When n Is Infinite.

From equation (2), we find that

$$D_x P_n = a^{n-1} e^{-xa^{n-1}} - a^n e^{-xa^n}$$

setting this expression equal to zero, simplifying, and writing X_n for x, we find

$$X_n = \frac{-\log_e a}{a^{n-1}(1-a)}$$

After substituting this expression for x in equation (2), and simplifying, it appears that

$$P_\infty = a^{\frac{a}{1-a}} - a^{\frac{1}{1-a}} \tag{4}$$

The value of P_∞ given in Column 3 was found by setting a equal to .1 in equation (4).

The Value of p'_n for Small Values of n.

Between X_n and X_{n+1}, there must be a value of x for which $p_n = p_{n+1}$. We denote this value by X'_n. When $x = X'_n$, the degree of consistency of titrations of samples containing the same number of particles will have a minimum value inasmuch as it is equally probable that a run will yield either n or $n+1$. The value of p_n corresponding to X'_n will be denoted by p'_n. We proceed to find the values of X'_n. Setting the expression given by equation (1) for p_n equal to a similar expression for p_{n+1}, rearranging terms, and writing X'_n for x, we have

$$\left[\frac{1 - a^n}{1 - a^{n-1}}\right]^{X'_n} \left\{2 - \left(\frac{1 - a^{n+1}}{1 - a^n}\right)^{X'_n}\right\} = 1 \tag{5}$$

Equation (5) shows that $X'_1 = 7.27$. For higher values of n, the equation cannot be solved for X'_n explicitly, but the values of X'_n can be found to any desired degree of approximation as follows: We set the quantity inside the brackets equal to zero, thus—

$$X'_n = \frac{\log 2}{\log (1 - a^{n+1}) - \log (1 - a^n)} \tag{6}$$

Having found a value of X'_n for any small value of n from equation (6), we use this value as the exponent of the first parenthesis of equation (5). This gives a new value of the quantity inside the brackets slightly different from zero, and consequently a new equation like equation (6) except that the figure 2 is replaced by a quantity slightly less than 2. This process could be carried on indefinitely but inspection shows that the true final value of X'_n cannot differ from the value first found from equation (6) by as much as .1 of 1 per cent for any value of n. We, therefore, use equation (6) and ignore the error in-

volved. Column 4 of Table I contains the values of X'_n thus found and Column 5 contains the corresponding values of p'_n found by substituting the values in Column 4 together with the corresponding values of n in equation (1). Inasmuch as X'_n must, in fact, be an integer, these values are slightly too small. The error is certainly negligible if n is 2 or more.

The Value of p'_n When n Is Infinite.

To make sure that p'_n approaches a limiting value as n increases, we find the value of p'_n when n is infinite. As in the preceding section, we first set p_n equal to p_{n+1} to find X'_n. Using equation (2) for the purpose, writing X'_n for x, and introducing a new variable, y, such that

$$X'_n = \frac{\log_e y}{a^{n-1}(1-a)}, \tag{7}$$

we find that

$$y(2 - y^a) = 1 \tag{8}$$

Equation (8) is the analogue of equation (5), and the value of y can be found by the same method of approximation. Inspection shows, as before, that

$$y = 2^{\frac{1}{a}} \tag{9}$$

gives log y with a maximum error of .1 of 1 per cent. Substituting this value of y in equation (7), we have

$$X'_n = \frac{\log_e 2}{a^n(1-a)} \tag{10}$$

and this expression when substituted in equation (2) gives

$$p'_\infty = 2^{\frac{1}{a-1}} - 2^{\frac{1}{a(a-1)}} \tag{11}$$

p'_∞ is, therefore, equal to .463 with an error of less than .1 of 1 per cent.

The Value of $\bar{p}_n$ for Small Values of n.

If we are working with solutions of a great variety of degrees of concentration, we are justified in considering the mean value of p_n for the values of x which lie between X'_{n-1} and X'_n. This quantity is denoted by $\bar{p}_n$. If n is greater than 1, we may treat x as a continuous variable without introducing an appreciable error. We simply integrate $p_n dx$ (using equation (1) for the purpose) between the limits X'_{n-1} and X'_n, and divide by the difference of the limits. We find, thus, that

$$\bar{p}_n = \frac{1}{X'_n - X'_{n-1}} \left[\frac{(1-a^n)^{X'_n} - (1-a^n)^{X'_{n-1}}}{\log_e (1-a^n)} - \frac{(1-a^{n-1})^{X_n} - (1-a^{n-1})^{X'_{n-1}}}{\log_e (1-a^{n-1})} \right] \tag{12}$$

The values of $\bar{p}_n$ for $n = 2$ and $n = 3$ shown in Column 6 were found by substituting the figures of Column 4 together with the appropriate values of n in equation (12).

The Value of $\bar{p}_n$ When n Is Infinite. ·

As before, we integrate $p_n dx$ from X'_{n-1} to X'_n (using equation (2)) and divide by the difference of the limits. X'_n is given by equation (10) and X'_{n-1} is a similar expression with the value of n reduced by one unit. After integrating, substituting these expressions for the limits, and simplifying, it comes out that

$$\bar{p}_\infty = \frac{1}{\log_e 2} \left[2^{\frac{a}{a-1}} + a \cdot 2^{\frac{1}{a(a-1)}} - (1+a)\, 2^{\frac{1}{a-1}} \right] \tag{13}$$

which proves to be .602.

The Effect of Altering the Dilution Factor.

In the preceding pages, a has been taken as .1. Increasing the value of a would result in a lowering of the values of the various p's; and conversely a decrease in the value of a would have the opposite

effect. To make sure that a slight change in the dilution factor could not produce a great change in the results, I have recalculated P_n and p'_n taking .09 for a. The results are shown in Columns 7 and 8. If we set a equal to zero in equation (13), we find that the limiting value of $\bar{p}_\infty$ is .722.

DISCUSSION.

In the foregoing, it has been necessary to deal with x as a continuous variable and to consider the case in which x is infinite. One must be careful not to confuse these analytical devices with the idea that the active substance is itself infinitely divisible; they were used simply for the purpose of studying equations (1) and (2) which are based on the particulate hypothesis. The low values of the p's in Table I are brought about by the fact that, however nicely the active substance may be divided by serial dilution in the first stages where the number of particles per cc. is great, a time comes as the dilution continues when the number of particles per cc. is so small that the probability variations are considerable. It is by the indications at this point that the state of affairs in the first tube is judged.

In practice, n is much greater usually than 1 or 2. We may, therefore, ignore these two cases. We take a as .1. Table I shows that a value 10 per cent lower makes little change in the results; we may, therefore, ignore the effects of slight errors of dilution.

The table shows that if $n > 2$ all of the p's are practically independent of n. It makes little difference, then, whether a particular tube, (the first tube as we have taken it) receives exactly x particles, whether it is made from a parent tube the concentration in which is ten times as great, or whether it is merely a sample of stock solution.

It appears from Column 3 that, with a fortunate choice of the solution to be titrated, about 70 per cent of parallel runs might yield the same value of n. On the other hand, if the choice were unfortunate, less than half of them would yield the same value of n. In the long run, working with a great variety of solutions, we should expect 60 per cent to yield the same value. The discrepancy between this figure and Dr. Bronfenbrenner's estimate, 85 per cent, based on the actual yield of the method in practice is, in Dr. Bronfenbrenner's opinion, too great to be ignored.

It will be remembered that our analysis of the problem was based on the simple assumption that only one particle need be put into a tube in order to dissolve the bacteria in it. It has not been assumed that the particles are alike. The particles may be molecules—all alike—or they may consist of particles of foreign matter on the surfaces of which one or more of the ultimate units of bacteriophage have been adsorbed. We have required only that particles neither divide nor coalesce during the process of dilution (only the second of these processes would make $\bar{p}_n$ greater). It is, of course, conceivable that, in concentrated solution, a change of concentration might have some influence on such particles, but it is hard to imagine how any such change could take place during the process of serial dilution after a point has been reached where there are only from one to ten particles in 10 cc. of broth. Such changes in the first part of the series would have a profound effect on the accuracy of estimates made by the method, but none on the degree of consistency of the results.

It is conceivable that the interaction of a bacterium and a particle of bacteriophage is, in itself, a matter of probability. The particle may be inactive, or it may attach itself to a bacterium which is not susceptible. It is reasonable to assume that, of the whole number of bacteria added to each tube, a constant fraction are susceptible. We may say, then, that there is a certain constant probability, q, that any particular unit of bacteriophage will act effectively. This could have been taken into account very easily in deriving equations (1) and (2), thus—if, instead of considering the probabilities, a^{n-1} and a^n, that a particular unit of bacteriophage would be transferred to the nth and $(n+1)$th tubes respectively, we had considered the probabilities that the particular unit would act effectively in these two tubes, we should have found them to be $a^{n-1}q$ and a^nq respectively. q may now be replaced by some unknown positive power of a. It is evident, therefore, that the effect of introducing q is to increase the value of n. This means that the limiting values of the p's remain unchanged and that the values of the p's for small values of n, are, for the same value of n, more nearly in coincidence with the limiting values than they would be if q were not introduced; in other words, if n is greater than 2, the introduction of q is without appreciable effect.

We have next to consider adsorption losses. During the process of

stirring and transferring fluid, some of the particles must come in contact with the surfaces of the tube and the pipette and it may be that some or all of them adhere to the glass. Although this would not necessarily render the particles inactive, it would effectively prevent transferring them to the next tube. Such losses, if they exist, must be very small. Dr. Bronfenbrenner[1] has found that a very dilute solution (corresponding approximately to Tube $n - 2$) gives the same value of n whether it is titrated immediately after preparation or after having been kept in glass for 72 hours. This means that, during the 10 minutes required to make a transfer, only a very small fraction, certainly much less than 10 per cent, of the whole number of particles in the tube will be adsorbed. Since the fraction is so small, and since the transfers to successive tubes require about the same length of time, we may say that there is a definite probability, which is the same for all of the transfers, that any particular unit of bacteriophage, which has been transferred to any tube, will escape adsorption until the transfer of fluid to the next tube has been made. This probability may be combined with the dilution factor, a, to give a new and slightly smaller value of a. If 10 per cent of the particles were lost at each transfer, a would be reduced from .1 to .09. Table I shows that the corresponding increases in the values of the p's amount to only 2 or 3 per cent.

If, therefore, it is true that when one active particle of bacteriophage comes in contact with a susceptible bacterium, all of the bacteria in the tube dissolve, we are justified in expecting that, in the long run, about 60 per cent of parallel runs will yield the same value of n. This figure will remain unaltered whatever value we assign to the probability either that a particle is by nature inactive, or that it is taken up by a bacterium which is not susceptible; and it will change only slightly as a result of the greatest adsorption losses which we have reason to consider.

If experiment should show definitely that the serial dilution method yields results with a degree of consistency much greater than 60 per cent, the most obvious explanation of the discrepancy will be that one particle is not usually sufficient to cause the dissolution of all of the bacteria in the tube, even though it is active and comes in contact with a susceptible bacterium. This idea is not seriously in conflict

with the most important feature of the particulate hypothesis as usually understood, *i.e.* that one particle can start the process of dissolution. It is conceivable that a single infected bacterium may not be able to produce enough particles of bacteriophage to infect all of the others within the time during which the bacteria remain susceptible.

SUMMARY.

1. The theory of the serial dilution method of titration of bacteriophage has been worked out on the basis of the simple particulate hypothesis.

2. It has been shown that, if the dilution constant is .1, only about 60 per cent of parallel runs on the same solution should give the same end-point, the average being taken over a great number of titrations of each of a great variety of solutions.

3. The discrepancy between this figure, 60 per cent, and Dr. Bronfenbrenner's estimate, 85 per cent, is considerable.

4. Inasmuch as the particulate hypothesis is well founded, no explanation of the discrepancy is suggested.

[Reprinted from the JOURNAL OF MORPHOLOGY AND PHYSIOLOGY, June 5, 1927, Vol. xliv, No. 1, pp. 117–125]

PARENTAL CHROMOSOME DIMENSIONS IN ASCARIS.

A STUDY OF THE EFFECT OF CELLULAR ENVIRONMENT ON CHROMOSOME SIZE.

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AUTHOR'S ABSTRACT.

In the early cleavage stages of Ascaris the homologous chromosomes are of unequal length. Measurements show that these homologues fall into two sharply defined groups suggesting their biparental origin. The shorter are considered to have come from the male.

As the age of the embryo increases, these differences between the chromosome mates tend to become less, and it is suggested that at some later period in the history of the animal this difference will entirely disappear in response to the effect of continued existence in a common environment The length of the chromosomes is very slightly shortened during the early cleavage divisions, while the area of the equatorial cross-section of the cells becomes enormously reduced.

Earlier observations tended to indicate strongly the fundamental character as well as the constancy of the size relations of the chromosome pairs in any particular species. It is, of course, entirely possible that these dimensions may have no more profound significance than being merely hereditary 'carry overs' from generation to generation. It is difficult, however, after an extensive study of these conditions, not to be impressed with the exactness of the interpair length relationship and with its maintenance under all variations in the absolute size of the individual pairs. Speculation as to the meaning or value in the life of the cell or organism of this metrical constancy very soon proves futile and turns one back to the further investigation of the more obvious problems involved.

The small size and relatively large numbers of the chromosomes measured in previous studies (pig, oenothera, chick, 1, 2, 3) leaves something to be desired in drawing conclusions on chromosome size variation limits. The need of standards of chromosome variation the accuracy of which would be entirely satisfactory led the writer to a study of the cells of the developing embryos of the round-worm

parasite of the horse, Ascaris megalocephala, the chromosomes of which are so few in number, so large in size, and can be found lying so nearly in one plane of focus that they seemed eminently suited to the purpose. A large number of metaphase chromosome plates have been drawn and measured with interesting and, at first sight, surprising results quite aside from the original purpose of the investigation.

MATERIAL AND METHODS.

I was fortunate in being loaned by Mrs. C. E. McClung many excellent preparations of the uterus of Ascaris sectioned through the region where first and second cleavages were common. When it became evident that later stages were also desirable, Dr. E. E. Carothers most kindly sent me a number of prepared slides as well as embedded material containing the needed embryonic stages. All of the material had been fixed in 90 parts of 70 per cent alcohol and 10 parts of glacial acetic acid, embedded in paraffin, sectioned at 10 μ, and stained with iron haematoxylin. The preparations are very beautiful, and the great numbers of dividing cells gave excellent opportunity for the selection of the best division figures.

Only those chromosomes were drawn where the entire length of the chromosome lay in one plane of focus or so nearly so that its tilt was negligible. Without this care in selection, the end results would, naturally, mean little. Although it was originally planned to draw each set of chromosomes at least twice, as done with the chromosomes of other forms (1, 2, 3), it was found that the great size and perfect separation of the Ascaris chromosomes made the tracing of them under the camera lucida so easy that the accuracy of the first drawing could not be improved upon, and consequently the repetition of the drawings was discontinued.

A pair of dividers with the points set 2 mm. apart was used in spacing off the length of the chromosomes, as was done in the previous studies referred to. A planimeter was employed to determine the area of the equatorial cross-sections of the various cells.

The chromosomes of 130 cells have been drawn and measured—sixty-five cells of the first cleavage division, fifty-seven cells of the second cleavage, and eighteen from the blastulae. The last are, of

course, less numerous, as in only one cell of the aggregation—the primordial sex cell—are the chromosomes whole. The fragmented chromosomes of the somatic cells, naturally, are useless for the purposes of this investigation.

The chromosomes were drawn at table level, using a Zeiss 1.5-mm. apochromatic objective and a Zeiss 10 × compensating ocular.

OBSERVATIONS.

The lengths of the chromosomes in the first- and second-cleavage stages.

Table 1 presents in tabular form the average lengths of the chromosomes found in a number of cells of early embryos of Ascaris. While these figures are averages, they nevertheless represent very fairly the measurements of the chromosomes of individual cells and can be accepted as quite typical of the usual conditions.

The homologues of each pair are in no case of similar length, which, in view of our general conception of their usual equality, at first seems surprising. That this variation is not accidental, due to technical errors, is shown by the length differences between the longer and shorter members of each of the two pairs (between chromosomes 1 and 2 and 3 and 4), being practically identical (10 to 12.5 per cent). Furthermore, the length variation between the longer chromosomes of each pair (chromosomes 1 and 3) and the shorter chromosomes of the same homologues (chromosomes 2 and 4) is even more nearly the same. Chromosome 3 is in all cases 20 per cent shorter than chromosome 1, while chromosome 4 varies by the same amount from chromosome 2 in the case of the chromosomes of the second cleavage and is but 22 per cent shorter than chromosome 2 of the first cleavage division. These measurements and comparisons indicate beyond question that there are two distinct groups of chromosomes in these cells—one group made up of chromosomes 1 and 3 and the other of chromosomes 2 and 4.

The length of the chromosomes in later cleavage stages.

The material available showed no stages later than the blastula which could not, from its general appearance, have been the product of more than four to five cell divisions. The chromosomes in these

cells have therefore been associated for from two to four cell generations longer than those recorded above. From table 1 it can be seen that the lengths of the mates are much more nearly equal than they were in the younger stages.

TABLE 1

Presents the average length in millimeters of the various chromosomes of the Ascaris complex. The length difference in per cent by which the shorter member of each pair varies from the longer is given in italics. The shorter member of each pair was presumably contributed by the male

Pair number	1		2		Number of cells measured	Total length of chromosomes	Area
Chromosome number	1	2	3	4			
Average length, first-cleavage chromosomes	36 5	32	28.5	25	65	121	33 4
Length of difference in per cent	*10*		*12 5*				
Average length second-cleavage chromosomes	36	32.5	29	26	57	123.5	19.5
Length difference in per cent	*10*		*11*				
Average length, blastula chromosomes	28	26	23	22	18	99	9 5
Length difference in per cent	*7*		*5*				

The area of the equatorial cross-section of the cell in relation to the total length of the chromosomes within it.

Table 1 gives the average areas of the equatorial cross-sections of forty-two cells. It is, of course, impossible to be certain that the observer has outlined the exact equator of the cell wall, but as the chromosomes divide very near to the center of the cell, when they are present and in focus, it is very probable that the outer visible limits of the cell are not much on one or the other side of the equator. That this is borne out by experience is demonstrated by the actual measurements of the areas contained within the three classes of cells studied. In the cells of the first and second cleavage divisions the areas of the individual cells vary by very little from the average of their group—

hardly more than could be caused by the normal differences between cells of the same generation. Greater variation is found between the single areas and the average area in the third group, as under this heading have been grouped stages of cleavage of uncertain number of divisions.

The area of the equatorial cross-sections of these various cleavage-cell groups is in general inversely proportional to the number of di-

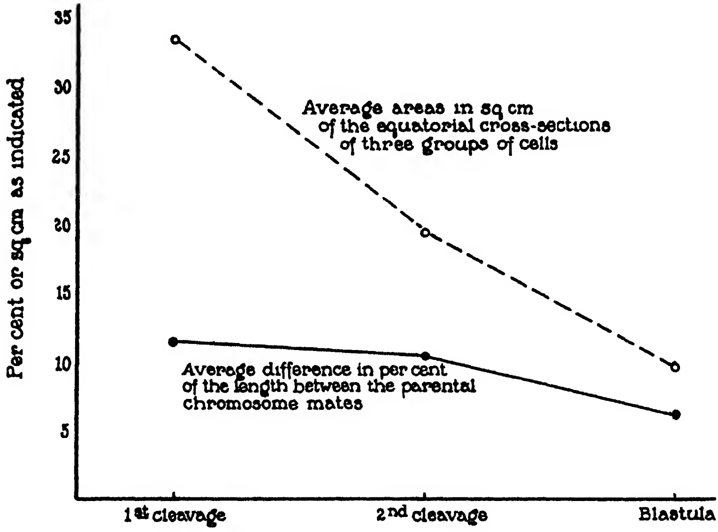

Fig. 1. The solid-line curve is based upon table 1, illustrating the decrease in the percentage of difference in the length of the parental chromosome mates with the increase of the time of their actual association in a common environment, i. e , within the same cells of the same organism. The broken line represents the decrease in area of the equatorial cross-section of the three groups of cells.

visions that have occurred. The more divisions, the smaller the area. The total length of the chromosomes, however, is not reduced with the same rapidity (fig. 2). While the shortest of the total chromosome lengths is 81 per cent of that of the chromosomes of the first and second cleavage divisions, the equatorial area of this same group of cells is but 29 per cent of that found in the largest group. Although two different units—length and area—have been compared above,

it may be pointed out that the length of the chromosomes is at least roughly proportional to their area and volume, and therefore is sufficiently satisfactory for this comparison.

DISCUSSION.

The considerable and constant difference between the lengths of the individual members of the chromosome pairs in the first and second cleavage divisions of Ascaris suggests the possibility of an initial variation in the size of the maternal and paternal contributions, the latter probably being the shorter. Logically, this seems reasonable. The chromosomes of the egg have been in an environment rich in nutriment and uncramped in space. The sperm chromosomes have been crowded into the smallest possible quarters. The first and second cleavage divisions occur so rapidly following fertilization that in all likelihood the male-cell chromosomes have little time to acquire much from their more favorable environment. If this reasoning is correct, the chromosomes of the later divisions should show progressively less difference between the lengths of the members of the pairs. It has been shown that an increased age of even a few cell generations has produced a considerable change in the relative lengths of the homologues, the members of which are found to be slowly approaching equality. It seems entirely probable that in still later embryonic stages we should find this equality of length had been reached.

On the basis of these observations, it seems justifiable to conclude that the parental chromosomes, as they appear in the first cleavage division of Ascaris, change their relative lengths as the embryo ages in response to their cellular environment. The interpair relationship —the percentage length difference between either the pairs of the chromosome series or between the individual members of one pair with the corresponding member of the next pair in the series—however, remains practically constant regardless of any absolute length changes of the chromosomes. This general behavior has been reported for several other forms (1, 2, 3).

The relation between the change in size of the chromosomes and the size of the cells during the early embryonic stages is interesting, in view of the very small change in the size of the chromosomes (19 per cent) and the enormous reduction in the area of the equatorial

cross-section. The lack of coincidence between cell and chromosome size is interesting, in view of the old kernplasm theory.

It may be well to anticipate possible misconceptions concerning the claims or scope of the above observations The problem as outlined is not concerned with the individuality of the chromosomes as in the case of the studies of Moenkhaus (4), Zoja (5), and others, although the data might well be placed in evidence as support for this theory.

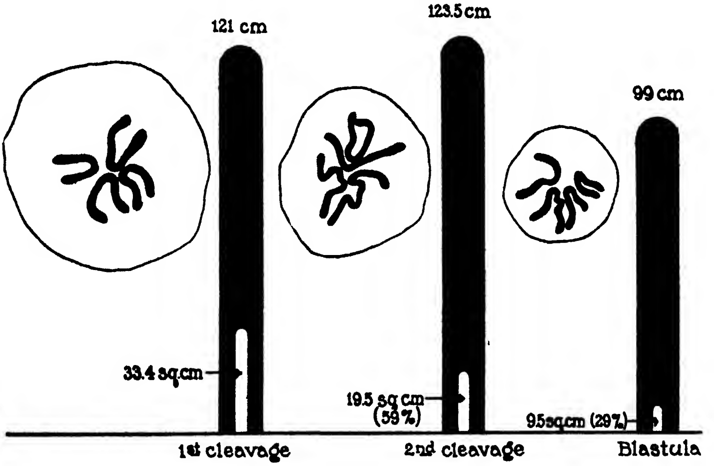

Fig. 2 The black columns represent the total length of the chromosomes in the three groups of cells described The white columns indicate the comparative areas of the equatorial cross-sections of these cells Note how much more rapidly the cell area has decreased than has the chromosome length. The percentages printed below the area values indicate the relation of that area to the area of the first cleavage cells. The outlined cells to the left of the black columns are drawings of representative cells of each class.

The reports of these investigators deal with the retention of chromosome identity in the offspring of species crosses in cases where the respective chromosomes were of recognizably different numbers or sizes. The development of these embryos was limited to a few cell generations indicating the incompatibility of the germinal mixtures. In such cases it would be most unlikely that the common environment of the zygote would produce any but a slowly destructive effect on the

hybrid sets of chromosomes or vice versa. The present account deals with the behavior of the parental chromosomes in normal fertilization and the gradual equalization of the lengths of the two sets of chromosomes that have come together. This is but a demonstration of what must obviously be a logical and theoretical necessity. The chromosomes respond to their environment within the limits of their inherent powers in early development and, as has been previously shown (1, 2, 3), continue to fluctuate more or less in size during the life of the organism. These claims for the dimensions of chromosomes in normal crosses, of course, have no bearing on what may happen in the cells of such viable hybrids as the mule, and it would, indeed, be of considerable interest to determine the metrical behavior of the chromosomes in such species crosses.

SUMMARY.

1. In the early cleavage stages of Ascaris the homologous chromosomes are of unequal length.

2. Measurements show that these homologues fall into two sharply defined groups suggesting their biparental origin. The shorter are considered to have come from the sperm.

3. As the age of the embryo increases, these differences between the chromosome mates tend to become less, and it is suggested that at some later period in the history of the animal this difference will entirely disappear in response to the effect of continued existence in a common environment.

4. The length of the chromosomes is very slightly shortened during the early cleavage divisions, while the area of the equatorial cross-section of the cells becomes enormously reduced.

5. These statements have no bearing on the behavior of the chromosomes in viable species crosses.

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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, June, 1927, Vol. lxxiii, No. 2, pp. 679–694.]

THE INFLUENCE OF SOLVENT AND OF CONCENTRATION ON THE OPTICAL ROTATION OF THE PENTACETATES OF GLUCOSE AND MANNOSE.

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(Received for publication, March 28, 1927.)

It has been known for a long time that the magnitude of the optical rotation of a given substance is a variable depending upon external conditions. Among these, concentration, solvent, and temperature are of major importance. Comparing the rotations of two epimeric substances containing in their molecules only 1 asymmetric carbon atom, it is found that the numerical values of the rotations of each epimer are identical for a given solvent and for a given concentration and that the differences are only in the direction of the rotation, one epimer rotating to the right and the other to the left. The question arises as to the behavior of substances with more than 1 asymmetric carbon atom, such as sugars. It was shown by Hudson[1] that the numerical values of the rotations of certain sugar derivatives may be regarded on the basis of van 't Hoff's superposition theory as the algebraic sum of the rotations of the individual carbon atoms. Hudson has based on this conception a method of differentiation between the α and β forms of sugars and Levene[2] later showed that the same conception may serve as a basis for a method of differentiating between the configurations of individual carbon atoms of a pair of epimeric sugar acids. Observations on the α and β forms of sugars have brought to light the exceptional behavior of some sugars, and in recent years Hudson[3] and also Levene[4] have made an effort to connect the abnormal optical behavior with the peculiarities of the lactal structure of the exceptional sugars. However, before proceeding

[1] Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66.

[2] Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 146

[3] Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1424.

[4] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, 759, 771.

further in this direction it seemed desirable to answer the following question: Are the rotations of each asymmetric carbon atom of a molecule influenced by a given solvent in the identical manner or does the influence vary from carbon to carbon atom depending upon the configuration of the entire molecule or upon the differences in ring structure? Some suggestion as to the possibility of individual influence of the solvent on each carbon atom may be found in the observation of Levene and Meyer[5] on the influence on rotation of the methylation of the individual carbon atoms of gluconic acid.

The present investigation deals with the influence of solvents and of concentrations on the rotations of the pentacetates of glucose and of mannose. These two sugars were selected for the reason that one (glucose) behaves normally according to the rule of Hudson and the second abnormally, whereas structurally they are a pair of epimers.

The rotation of glucose pentacetate has already been measured in several solvents by Hudson and Dale.[6] Their observations were limited to low concentrations. In the present investigation the concentrations were varied from about 2 to 80 per cent where solubility permitted.

The results are tabulated in Table I and are graphically represented by curves in Figs. 1 and 2. In Columns 1, 3, and 5 are given the concentrations in gm. per cc., the rotations being measured in tubes of 1, 2, and 4 dm., depending upon the sugar and the concentration. It was so planned that in no case was the total rotation less than 7°. The numerical values of observed rotations divided by the length of the tube are given in Columns 2, 4, and 6 of Table I.

These results are shown graphically in Figs. 1 and 2 where the concentrations as given in Table I are plotted as abscissæ and the rotations in degree per 1 dm. tube as ordinates.

From these curves, plotted on a large scale (commensurate with the experimental precision), the rotations at round concentrations were interpolated. The values thus obtained are given in Columns 2, 5, and 8 of Table II. The specific and the molecular rotations calculated from these values are recorded in the columns headed $[\alpha]$ and $[M]$ respectively.

[5] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1925, lxv, 535.

[6] Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1264.

TABLE I.

Optical Rotations of the α- and β-Pentacetates of Mannose and Glucose in Different Solvents and at Different Concentrations.

$t = 25.0° \pm 0.1°$ $\lambda = 5461$ Å $l = 1$ dm.

Chloroform.		Acetone.		Benzene.	
Concentration in gm. per cc × 10². (1)	α (2)	Concentration in gm. per cc. × 10². (3)	α (4)	Concentration in gm per cc. × 10². (5)	α (6)
A. α-Mannose pentacetate.					
	degrees		degrees		degrees
2.913	1.88	2.306	1.33	6.03	4.23
4.300	2.77	3.520	2.12	8.03	5.64
7.750	5.12	6.480	3.79	9.12	6 39
8.550	5.64	7.440	4.36	13.41	9.38
9.562	6.33	14.35	8.65	14.84	10.40
15.66	10 32	14 80	8.92	21.29	14.77
18.40	12 05	22 49	13.55	25.03	17.28
18.60	12 15	26 57	15 96	29 58	20 06
19.90	12.99	31 41	18 68		
24.10	15.30	33 29	19 64		
30 95	19.18	35 35	20.91		
45.80	27 60	49.98	28.76		
60 78	36.08	68 71	38 99		
80 33	47.16				
B. α-Glucose pentacetate.					
5.96	6 95	5 28	6.59	5 67	6.32
7 58	8 86	8 04	10 08	8 81	9.01
9 42	11.00	8.89	11.14	9 56	10 66
11.50	13 42	13 07	16.41	13 46	15.04
14 85	17 32	16 41	20.68	15.15	16 89
20.12	23 48	19 69	24.74	17.66	19.84
25.28	29 68	24 22	30 49	19 73	22 31
29.90	35 55	27 93	35 08	26 15	29 88
31.00	36 35	31 28	39 34	28 26	32 20
36 78	43 60	34 65	43 56		
43 19	51 36	38.67	48 33		
C. β-Mannose pentacetate.					
2.111	−0 59	5 054	−1 63	4 948	−1.77
9.944	−2.82	7 996	−2 61	7 142	−2.54
10 73	−3.05	11.27	−3 68	9.83	−3 49
22 86	−6 53	13.49	−4 42	15.56	−5 32
28 70	−8.19	20 96	−6 85	16 27	−5.54
37.37	−10 71	27.79	−9 04	20.01	−6 76
42.87	−12.35	34.70	−11.22	27.94	−9 33
58.77	−16.97	43 62	−14.07		
		44 89	−14.46		

TABLE I—*Concluded.*

Chloroform		Acetone		Benzene	
Concentration in gm per cc × 10²	α	Concentration in gm per cc × 10².	α	Concentration in gm per cc. × 10².	α
(1)	(2)	(3)	(4)	(5)	(6)
D. β-Glucose pentacetate					
	degrees		*degrees*		*degrees*
5 28	0 29	6 346	0 48$_5$	8.36	4.00
7 832	0 42$_5$	6 661	0 50	9.86	4 70
10 99	0 59	9 67	0 73	12 05	5 07
14 87	0 80	14 93	1.15		
18 70	0 99$_5$	18 39	1 46		
31 26	1 75	19 24	1 53		
35 10	2 02	24 79	2 01		
41 69	2 48	26 82	2 14		
46 19	2.80	31 59	2 61		
Glacial acetic		**Methyl alcohol**		**Pyridine**	
Concentration in gm. per cc × 10²	α in degrees per 1 dm tube	Concentration in gm per cc × 10²	α in degrees per 1 dm tube	Concentration in gm per cc × 10²	α in degrees per 1 dm tube
E α-Mannose pentacetate.					
7 492	4 75	7 102	4 54	6 700	3 76
8 432	5 30	8 352	5 36	8.265	4 64
9 281	5 84	9 701	6 21	9 325	5 24
α-Glucose pentacetate.					
7 852	9 78	6 645	8 01	6 672	7 16
8 315	10 32	8 472	10 20	8.363	8 98
9 244	11 48	9 045	10 83	9 662	10 39
β-Mannose pentacetate.					
Concentration in gm per cc × 10².	α in degrees per 2 dm tube	Concentration in gm per cc. × 10²	α in degrees per 2 dm tube	Concentration in gm per cc × 10²	α in degrees per 2 dm tube
8 528	−5 32	6 796	−4 28	5 961	−4 72
9 610	−6 06	7.963	−4.86	7 009	−5 60
10 555	−6 68	9 601	−5 84	8 404	−6 65
β-Glucose pentacetate					
Concentration in gm per cc × 10²	α in degrees per 4 dm tube			Concentration in gm per cc × 10²	α in degrees per 4 dm tube
7.939	1 74			7 081	−0.97
8.007	1.74			8 097	−1 21
9 402	4 08			10 65	−1.45

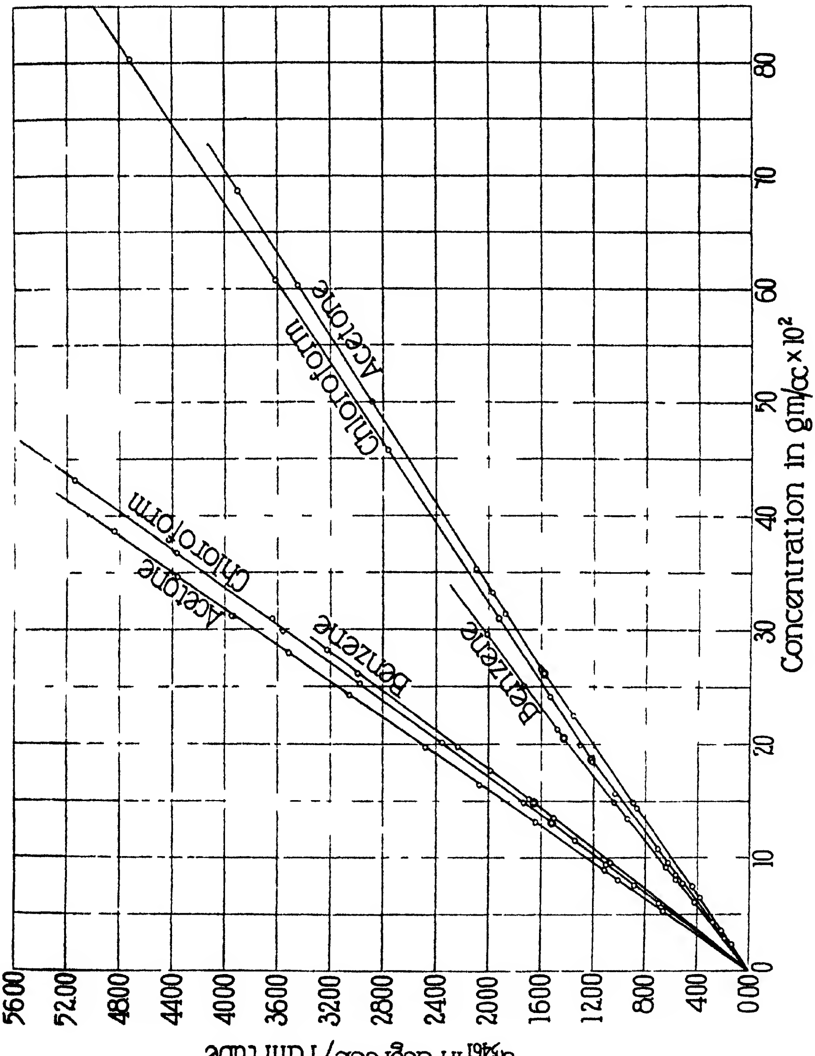

FIG 1 Optical rotations of the pentacetates of α-glucose (upper group), and α-mannose (lower group) in different solvents In Figs 1 and 2 the double circles indicate points of inflection; not all of these are experimentally determined points.

The differences between the α and β forms of the same sugar in the same solvent and concentration are given in Table III.

The analysis of the curves representing the rotations of the solutions as a function of concentration reveals certain very significant peculiari-

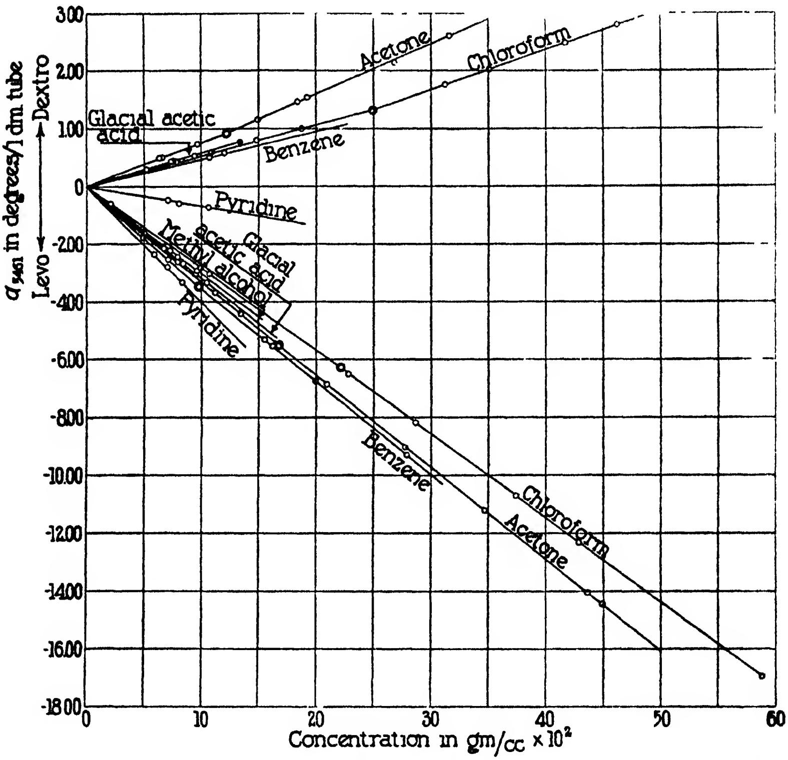

FIG. 2. Optical rotations of the pentacetates of β-glucose (upper five curves) and β-mannose (lower six curves) in different solvents.

ties. First, practically all the curves show at a certain concentration a break, after which the curve assumes a new slope. Second, the curves expressing the rotations in individual solvents do not run parallel to each other, but diverge with increase in concentration. Third, the specific rotations remain practically constant in dilute solutions and

TABLE II.

Rotatory Power at Round Concentrations.

25.0° ± 0.1° λ = 5461 Å l = 1 dm.

Concentration in gm per cc. × 10².	Chloroform			Acetone			Benzene		
	α	[M]	[α]	α	[M]	[α]	α	[M]	[α]
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
A. α-Mannose pentacetate.									
2 0	1 28	250$_0$	64 0	1 12	218$_4$	56 0	1 40	273$_0$	70 0
4 0	2 50	243$_8$	65 5	2 34	228$_1$	58 5	2 81	274$_0$	70 3
6 0	3 90	253$_5$	65 0	3 57	232$_0$	59 5	4 21	273$_6$	70 2
8 0	5 22	254$_5$	65 3	4 77	232$_5$	59 6	5 62	274$_0$	70 2
10 0	6 52	254$_4$	65 2	6 00	234$_0$	60 0	7 07	275$_7$	70 7
12 0	7.86	254$_9$	65 5	7 21	233$_8$	60 0	8 41	272$_7$	70 7
14 0	9 17	255$_4$	65 5	8 42	234$_6$	60 1	9 82	273$_6$	70 1
16 0	10 45	255$_4$	65 3	9 62	234$_5$	60 1	11 20	273$_0$	70 0
18 0	11 69*	253$_3$	64 9	10 62	234$_5$	60 1	12 52	271$_3$	69 6
18 6	12 16	255.$_0$	65 4	11 20	234$_8$	60 2	12 96	271$_7$	69 1
20 0	12 95	252$_5$	64 7	12 03	234$_6$	60 1	13 95	272$_0$	69 8
20 4							14 22*	271$_3$	69 7
22 0	14 08	249$_6$	64 0	13 23	234$_5$	60 1	15 23	270$_0$	69 2
24 0	15 21	247.$_2$	63 4	14 44	234$_6$	60 2	16 52	268$_5$	68 8
25 0	15 72	250$_9$	62 8	15 04	234$_6$	60 2	17 14	267$_4$	68 5
26 1				15 75*	235$_2$	60 3			
30 0	18 62	242$_1$	62 0	17 84	231$_9$	59 5	20 36	264.$_4$	67 9
35 0	21 46	239$_1$	61 3	20 58	229$_3$	58 8			
40 0	24 26	236$_5$	60 6	23 31	227$_3$	58 3			
45 0	27.14	235$_2$	60 3	26 05	225$_8$	57 9			
50 0	29 96	233$_7$	59 9	28 78	224$_5$	57 6			
60 0	35 65	231$_7$	59 4	34 21	222$_4$	57 0			
70 0	41 31	230$_2$	59 0						
80 0	46 90	228$_6$	58 6						
B. α-Glucose pentacetate.									
2 0	2 30	448$_5$	115 0	2 50	487$_6$	125 0	2 23	434$_3$	111.5
4.0	4 67	455$_3$	116 7	5 00	487$_6$	125 0	4.46	434.$_3$	111.5
6 0	7 03	456$_9$	117 2	7 56	487$_5$	125 0	6 69	434$_3$	111 5
8 0	9 37	456$_8$	117 1	10 03	488$_9$	125 3	8 94	435$_3$	111 7
10 0	11 68	455$_1$	116 8	12 54	489$_1$	125 4	11 16	435$_2$	111.6
12 0	14 00	455$_0$	116 6	15 06	489$_4$	125 5	13 38	434$_3$	111 5
13 05	15 20*	454$_2$	116 5						
14 0	16 31	454$_3$	117 0	17 59	490$_0$	125 6	15 61	434$_3$	111 5

* The concentration at which there is an inflection in the curve.

TABLE II—*Continued.*

Concentration in gm. per cc × 10²	Chloroform.			Acetone.			Benzene.		
	α	[M]	[α]	α	[M]	[α]	α	[M]	[α]
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
B. α-Glucose pentacetate—Concluded.									
16 0	18 68	455_{3}	116 7	20 14	490_{9}	125 9	17 88	435_{3}	111 7
18 0	21 04	455_{9}	116 8	22.64	490_{5}	125 8	20 25	$438._{7}$	112.5
20 0	23 40	456_{3}	117 0	25 14	490_{2}	125 7	22 60	440_{7}	113 0
22 0	25 79	457_{2}	117 2	27 64	490_{0}	125 6	24 93	441_{9}	113.3
24 0	28.20	458_{1}	117 5	30 15	489_{9}	125 6	27 27	443_{1}	113.6
26 0	30 60	459_{0}	117 7	32 68	490_{2}	125 7	29 60	444_{0}	113 8
28 0	33 00	459_{6}	117 9	35 18	490_{0}	125 6	31 90	444_{3}	113 9
30 0	35 42	460_{4}	118 1	37 66	489_{6}	125 5			
35 0	41.40	461_{3}	118 3	43 92	489_{4}	125 5			
39 0	46 29	462_{9}	118 7	48 96	489_{8}	125 5			
44 0	52 30	463_{6}	118 9						
C. β-Mannose pentacetate.									
3 0	0 85	110_{5}	28 3	0 96	124_{3}	32 0	1 08	140_{4}	36 0
5 0	1.41	110_{0}	28 2	1 62	126_{4}	32 4	1 79_{6}	139_{6}	35 8
7.0	1 97	109_{3}	28 1	2 28	127_{3}	32 6	2 48	138_{5}	35 5
9.0	2 54	110_{1}	28 2	2 95	127_{8}	32 8	3 20	138_{7}	35 5
9.9							3 48*	137_{1}	35 2
12 0	3 40	110_{5}	28 3	3 93	$127._{7}$	32 7	4 18	135_{9}	34 8
15 0	4.26	110_{3}	28 4	4 94	128_{4}	32 9	5.15	133_{9}	34 3
16 8				5 52*	128_{1}	32 6			
18 0	5 09	110_{3}	28 3	5 90	$127._{8}$	32 7	6 11	132_{4}	33 9
21 0	5 94	110_{3}	28.3	6 87	127_{6}	32.6	7 08	131_{5}	33 7
22.2	6 29*	110_{5}	28 3						
24 0	6 82	110_{8}	28 5	7 84	127_{4}	32.7	8 06	131_{0}	33 6
27 0	7 69	110_{8}	28 5	8 82	127_{4}	32 7	9 03	130_{4}	33 4
30 0	8 57	111_{4}	28 6	9.75	126_{7}	32 5	10 01	130_{1}	33 4
35 0	10.02	$111._{6}$	28.6	11.32	126_{1}	32 6			
40.0	11 49	112_{0}	28 7	12 90	125_{8}	32 2			
45 0	12 94	112_{1}	28 7	14 50	125_{7}	32 2			
50 0	14 40	112_{3}	28 8						
55 0	15 86	$112._{5}$	28 8						
D. β-Glucose pentacetate.									
2 0	0 12_{0}	23 4	6 0	0 15_{0}	29 2	7 5	0 09_{5}	18 5_{0}	4.7
4.0	0.22_{5}	21 9	5 6	0 30_{2}	29 4	7 5	0 19_{0}	18 5_{0}	4 7
5 0	0 28_{0}	21 8	5.6	0 37_{5}	29 2	7 5	0 24_{0}	18 7_{0}	4 8

TABLE II—*Continued.*

Concentration in gm. per cc. × 10². (1)	Chloroform. α (2)	Chloroform. [M] (3)	Chloroform. [α] (4)	Acetone. α (5)	Acetone. [M] (6)	Acetone. [α] (7)	Benzene. α (8)	Benzene. [M] (9)	Benzene. [α] (10)
D. β-Glucose pentacetate—Concluded.									
7.0	0 38$_5$	21 4	5 5	0 52$_5$	29 2	7 5	0 33$_5$	18 6$_8$	4 9
9 0	0 48$_0$	20 8	5 3	0 68$_0$	29 4$_7$	7 5	0 43$_0$	18 6$_3$	4 8
10 0	0.54$_0$	21.0	5 4	0 75$_0$	29 2	7.5	0 48$_0$	18 7$_2$	4.8
12.0	0 64$_8$	21 0	5 4	0 90$_0$	29 2	7 5	0 57$_5$	18 6$_9$	4 8
12.2				0 92$_0$*	29 4	7 5			
14 0	0 74$_8$	20 8	5 3	1 07$_1$	29 8	7 6	0 66$_5$	18 5$_2$	4.7
16 0	0 85$_5$	20 8	5 3	1 24$_4$	30 3	7 8			
18 0	0 96$_0$	20 8	5 3	1 42$_0$	30 7$_7$	7 9			
20 0	1 06$_0$	20.6$_7$	5 3	1 59$_9$	31 1$_8$	8 0			
22 0	1 16$_0$	20.5$_6$	5.3	1 77$_5$	31 4$_7$	8 1			
25 0	1 32$_0$*	20.5$_9$	5 3	2 03$_5$	31 7$_5$	8.1			
27 0	1 45$_5$	21 0$_2$	5 4	2 21$_0$	31 9$_2$	8 2			
29 0	1 59$_8$	21 4$_5$	5 5	2 38$_2$	32 7$_8$	8 2			
32 0	1 80$_8$	22.0$_0$	5 6	2 64$_0$	32 9$_2$	8.3			
35 0	2 02$_0$	22 5$_1$	5 8						
40 0	2 36$_2$	23 0$_3$	5 9						
45 0	2 71$_3$	23 5$_1$	6 0						

Concentration in gm per cc. × 10² (1)	Glacial acetic acid α (2)	Glacial acetic acid [M] (3)	Glacial acetic acid [α] (4)	Methyl alcohol α (5)	Methyl alcohol [M] (6)	Methyl alcohol [α] (7)	Pyridine α (8)	Pyridine [M] (9)	Pyridine [α] (10)
E. α-Mannose pentacetate.									
2.0	1 24	241 8	62 0	1 29	251 5	64 5	1 12	218 4	56 0
4 0	2 51	244 5	62 7	2 56	249 6	64 0	2 24	218 4	56 0
6 0	3 76	240.6	61 7	3 84	249 6	64 0	3 37	218 8	56 1
8.0	5 04	245.7	63 0	5 12	249 6	64 0	4 48	218.4	56 0
10 0	6 28	244 9	62 8	6 40	249 6	64 0	5 61	218 8	56.1
α-Glucose pentacetate.									
2 0	2.48	483 6	124.0	2 40	468 1	120 0	2 13	417.3	107.0
4.0	4 96	483 6	124.0	4 81	468 1	120 0	4 28	417 3	107.0
6 0	7 45	483.6	124 0	7 22	468 1	120 0	6.43	417 3	107.0
8.0	9.93	483.6	124.0	9 64	468.1	120 0	8 58	417.3	107.0
10 0	12 42	483.6	124 0	12.08	471 9	121 0	10 65	417.3	107.0

TABLE II—*Concluded.*

Concentration in gm. per cc. × 10². (1)	Glacial acetic acid α (2)	[M] (3)	[α] (4)	Methyl alcohol α (5)	[M] (6)	[α] (7)	Pyridine. α (8)	[M] (9)	[α] (10)
				β-Mannose pentacetate.					
2 0	$-0\ 62_8$	121 3	31 1	$-0\ 60_8$	$118\ 5_6$	30 4	$-0\ 79_5$	154 8	39.7
4 0	-1.25_6	122 4	31 4	$-1\ 20_1$	$117\ 0_0$	30 0	$-1\ 58_2$	154 4	39.6
6 0	-1.89_5	123 2	31 6	$-1\ 82_7$	118.5_6	30.4	-2.37_5	154 4	39.6
8 0	$-2\ 53_0$	123 2	31 6	$-2\ 43_5$	$118\ 5_5$	30 4	$-3\ 16_2$	154 1	39 5
10 0	$-3\ 16_3$	123 2	31 6	$-3\ 05_0$	$118\ 9_5$	30 5	$-3\ 96_0$	154 4	39 6
				β-Glucose pentacetate.					
2 0	$0\ 22_1$	42 90	11 0				$-0\ 13_5$	26.13	6 7
4 0	$0\ 44_0$	42 90	11 0				$-0\ 26_2$	25 74	6 6
6 0	$0\ 66_2$	42 90	11 0				$-0\ 40_5$	26.13	6 7
8 0	$0\ 88_0$	42 90	11 0				$-0\ 54_3$	26 52	6 8
10 0	$1\ 10_0$	42 90	11 0				$-0\ 69_2$	26 91	6 9

change markedly with increase in concentration. This point is shown in Fig. 3 where the molecular rotations are plotted as ordinates and the concentrations as abscissæ. Fourth, the numerical values of the specific rotation as a function of the solvent can be arranged in the following order.

A. For the α and β forms of glucose pentacetate the order is: acetone > glacial acetic acid > methyl alcohol > chloroform > benzene > pyridine.

B. The order for the pentacetates of mannose is, for the α form: benzene > chloroform > methyl alcohol > glacial acetic acid > acetone > pyridine; for the β form; chloroform > methyl alcohol > glacial acetic acid acetone > benzene > pyridine.

In this connection emphasis should be placed on the fact that the order of solvent influence on rotation in the mannose pentacetates is different from that in the two glucose pentacetates, and furthermore, that it is different in each of the two mannose pentacetates. In the case of the two α forms, the order is the same, but in the opposite sense as shown in Fig. 4. An exceptional position is occupied by pyridine. In this solvent all forms show the lowest rotations.

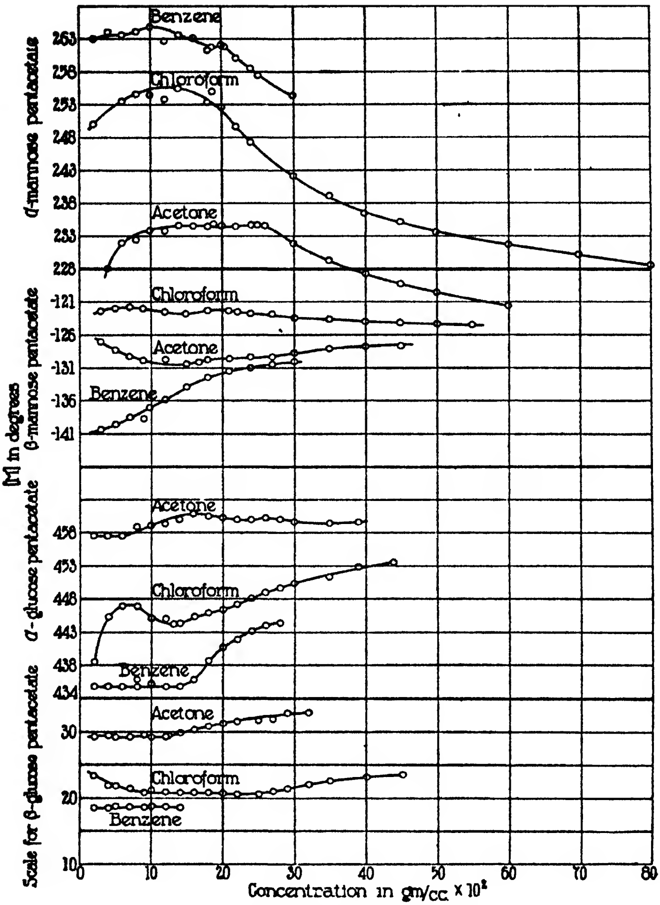

FIG. 3 Molecular rotations of the pentacetates of α- and β-glucose and of α- and β-mannose in different solvents.

α-Glucose	pentacetate	in benzene	[M]	scale,	O.K.
"	"	" chloroform	"	"	add 10.
"	"	" acetone	"	"	" 30.
β-Glucose	"	" all solvents	"	"	O.K.
α-Mannose	"	" benzene	"	"	"
"	"	" acetone	"	"	subtract 2.
"	"	" chloroform	"	"	" 12.
β-Mannose	"	" acetone	"	"	O.K.
"	"	" chloroform	"	"	"
"	"	" benzene	"	"	add 10.

Each of the four observations has its own significance. From the first observation one may conclude that the relationship between solvent and solute changes with increase in concentration. As yet there do not exist sufficient data to offer a definite explanation of that

TABLE III.

Differences between $[\alpha]^{25}_{5461}$ of α- and β-Mannose Pentacetates and α- and β-Glucose Pentacetates at Round Concentrations.

Concentration in gm. per cc. × 10².	Mannose pentacetates $[\alpha]_\alpha - [\alpha]_\beta$			Glucose pentacetates. $[\alpha]_\alpha - [\alpha]_\beta$		
	Chloroform	Acetone.	Benzene.	Chloroform	Acetone	Benzene
3 0	92 3	89 3	106 0	110 2	117.5	106 8
5 0	93 7	91 7	106 0	111 4	117 5	106.8
7 0	93.6	92 1	105 7	111.6	117.6	106 8
9.0	93 4	92 6	105 7	111 7	117.8	106 9
10 0	93 4	92.8	105 6	110 4	117.9	106.8
12 0	93 8	92 8	104 9	110 3	118 0	106 7
14 0	93 8	93 0	104 3	111 7	118 0	106 7
16 0	93.7	93 0	104 3	111 4	118 1	
18 0	93 2	92.8	104 5	111.5	117 9	
20 0	93 7	92 7	103 6	111 7	117 7	
22 0	93 0	92 7	102 9	112.0	117 6	
24.0	91.9	92 9	102 4	112 2	117 5	
25 0	91.3	92 9	102 3	112 3	117.5	
30.0	90 7	92 0	101 3	112 5	117 3	
35 0	89 9	91 4		112.5	117 2	
40 0	89 3	90 5		112 8		
45 0	89 0	90 1		112 8		
50 0	88 7					
55 0	88 4					

	Mannose: Glacial acetic acid	Mannose: Pyridine	Glucose: Glacial acetic acid.	Glucose: Pyridine.
2 5	62 4	96 0	113 6	112.8
5 0	62 8	95.6	112 8	113 2
7.5	62 6	95 4	112 6	112.6
10.0	62 6	95.4	113 0	114 0

relationship. It may be of a chemical nature, the solvent forming a complex with the solute. The ratio of the complex molecules to the simple may change with the increase in concentration. It may also be assumed that the solvent is attached to different atoms or groups of the solute depending upon the concentration. In fact, it has been

shown that pentacetate of glucose forms a definite compound with benzene[7] and also with pyridine.[8] On the other hand, the influence

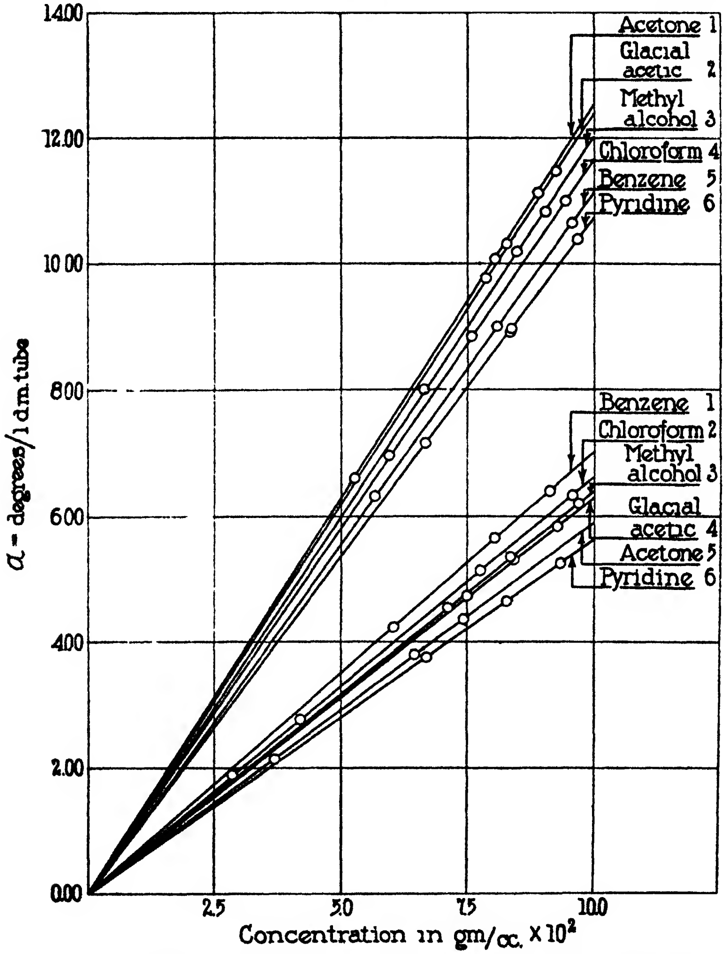

FIG. 4. The effect of solvents of the optical rotation of the pentacetates of α-glucose (upper group) and α-mannose (lower group).

[7] Moll van Charante, J., *Rec. trav. chim. Pays-Bas*, 1902, xxi, 42.
[8] Behrend, R., *Ann. Chem.*, 1907, cccliii, 106.

of the solvent may be entirely external, affecting the degree of deformation of the solute.

From the second observation it may be concluded that each solvent forms a different complex with the solute or that it brings about a different form of distortion of the molecule of the solute.

The third observation needs no special discussion in the light of the first two. However, it emphasizes a practical point; namely, that in the comparative study of the numerical relationships of the rotations of different sugars, those series of concentrations should be selected in which the specific rotations remain constant.

On the fourth observation centers the principal interest of the present investigation. It demonstrates that there exists a divergence in the influence of the solvents on the pentacetates of glucose as compared with the influence on the pentacetates of mannose. The differences are marked not only in regard to the respective specific rotations, but also in regard to the differences in the rotations of the α and β forms.

Hudson[6] and his coworkers have shown that with respect to the latter value the glucose pentacetates behave normally. In his latest publication Hudson[3] assigns to them the <1, 4> structure. In the same publication Hudson assigns to β-mannose pentacetate the <1, 4> structure and referring to the α form he remarks. "For the present the acetate of +55 rotation will be left unclassified; the determination of its ring form and even the question whether it may not be a mixture of substances *remain outstanding problems.*"[9] Levene and Sobotka,[4] on the other hand, on the basis of a comparative study of the rotations of the pentacetates of glucose, galactose, and mannose, were inclined to assign to the pentacetates of glucose the <1, 4> ring and to those of mannose the <1, 5> ring.

If a classification of pentacetates should be made on the basis of the order of influence of the solvent, the conclusion would necessarily be reached that the α- and β-glucose pentacetates belong to one type of structure, and that the α and β forms of mannose pentacetate belong to two different types, each distinct from that of the glucose pentacetates.

On the other hand, if the differentiation of the pentacetates should

[9] The italics are ours.

be made on the basis of the differences of the molecular rotations of the α and β forms, then one will be confronted with the following confusing facts.

The ratios of $\frac{[M]_\alpha - [M]_\beta \text{ glucose pentacetate}}{[M]_\alpha - [M]_\beta \text{ mannose pentacetate}}$ differ in individual solvents in the following way: chloroform 0.84, acetone 0.78, pyridine 0.85, glacial acetic acid 0.55, benzene 1.0.

Thus, if the conclusion was based on an observation in a single solvent the classification would depend on chance. In benzene the differences of the rotations of glucose pentacetate and of mannose pentacetate have the same numerical value; in glacial acetic acid the divergence is the greatest. In the other solvents the values are approximately in the middle of the distance between the two extreme values.

Thus, it seems suggestive that both the α and β forms of mannose pentacetate belong to a different type from that of the glucose pentacetates and it is also possible that the two forms of mannose pentacetate have ring structures differing from each other as was suggested by Hudson.

More information on the influence of various solvents on the rotations of epimeric sugar derivatives is needed before a final conclusion can be reached as to the structural relationships of the pentacetates of glucose and mannose. As yet, the problem is not definitely settled.

EXPERIMENTAL.

A Schmidt and Haensch polarimeter supplied with a large direct vision spectroscope as a monochromator was used in this work. A quartz mercury lamp served as a source of light. The green line 5461 Å was employed. The purity of this light, as well as the accuracy of the polarimeter, was tested by means of a quartz test plate recently calibrated at the Bureau of Standards. Jacketed tubes were employed throughout and a rapid stream of water from a regulated thermostat maintained a constant temperature of 25.0° ± 0.1°.

The solutions were made up by weighing the solids directly in 10, 15, or 25 cc. flasks. The solvent was then added up to the calibration mark. The flasks were carefully calibrated at 25.0°, and in making up the solutions the solvent was brought up to mark only after the

solution and the flask had been allowed to come to the proper temperature. Extra pains were taken in making up these solutions inasmuch as the total volume of solvent was small and a slight error in the volume made appreciable discrepancies in the final result.

Throughout this research the same sample of substance was used in the case of α- and β-glucose pentacetates and β-mannose pentacetate. In the case of the α-mannose pentacetate, however, we were obliged to use several samples. From these, only those giving the highest rotations were used.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1927,
Vol. lxxiv, No. 1, pp. 153–174.]

THE ROTATORY DISPERSION OF THE PENTACETATES OF α- AND β-GLUCOSE AND OF α- AND β-MANNOSE.

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(Received for publication, May 9, 1927.)

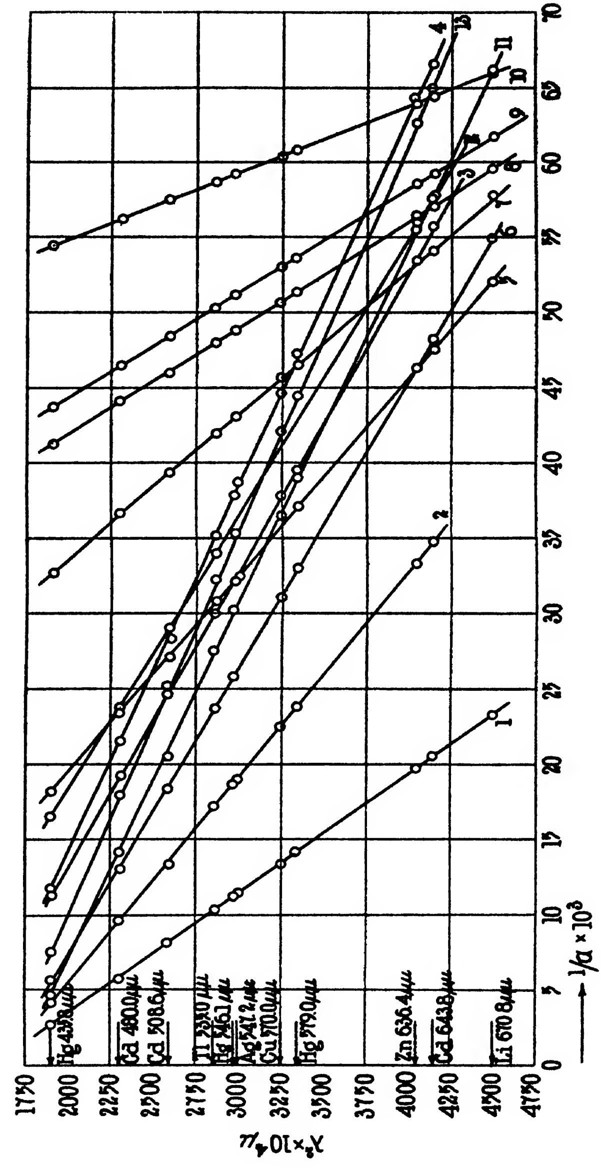
The van't Hoff superposition theory of optical rotation has been applied with much success by Hudson for the explanation of the optical properties of isomeric mono- and polysaccharides. The method of Hudson proved to be of great service, not only for the analysis of the optical rotations of known forms, but also for the prediction of the rotations of forms not yet known. However, in certain instances, namely in the cases of mannose, lyxose, and rhamnose, the rules of Hudson do not hold. The reasons for the deviations are as yet unknown.

The optical rotation of sugars is generally measured in the light of sodium vapor or in light of the same wave-length, the most convenient solvents being employed. Often only one solvent is used, frequently with few variations in the concentrations of the solutions. On the other hand, it is known that all these variables—wave-length, solvent, and concentration—affect individual substances in an individual manner.

The effects produced on the rotations of the pentacetates of α- and β-glucose and on the pentacetates of α- and β-mannose by different solvents and different concentrations have been discussed in a previous publication.[1] In the present communication the values are given of the optical rotations of the same pentacetates measured at ten different wave-lengths for different concentrations. The rotatory dispersion of the pentacetate of α-mannose has already been referred to in a previous communication.[2]

[1] Levene, P. A., and Bencowitz, J., *J. Biol. Chem.*, 1927, lxxiii, 679.

[2] Levene, P. A., and Bencowitz, J., *J. Biol. Chem.*, 1927, lxxii, 627.



Hg 435.8 μμ
Cd 480.0 μμ
Cd 508.6 μμ
Tl 535.0 μμ
Hg 546.1 μμ
Ag 547.2 μμ
Cu 570.0 μμ
Hg 579.0 μμ
Zn 636.4 μμ
Cd 643.8 μμ
Li 670.8 μμ
$\lambda^2 \times 10^4 \mu$
$1/\alpha \times 10^3$
1750
2000
2250
2500
2750
3000
3250
3500
3750
4000
4250
4500
4750
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
1
2
3
4
5
6
7
8
9
10
11
13

FIG. 1.

FIG. 1.

1. α-Mannose pentacetate in chloroform, concentration = 0 8033 gm. per cc Scale of $1/\alpha$, add 10.0.
2. " " " " " 0 4580 " " " " " " " 10 0.
3. " " " " " 0 3095 " " " " " " " 20 0.
4. " " " " " 0 2410 " " " " " " " 20 0.
5. " " " acetone " 0 4998 " " " " " " subtract 2.5.
6. " " " " " 0 3329 " " " " " " add 25 0.
7. " " " " " 0 6871 " " " " " " subtract 17 5.
8. " " " " " 0 07440 " " " " " " multiply by 10, subtract 300.
9. " " " " " 0 02306 " " " " " " " " 10, " 325.
10. " " " " " 0 03520 " " " " " " " " 10, add 475.
11. " " " " " 0 2674 " " " " " " add 32 5.
12. " " " chloroform " 0 1566 " " " " " " multiply by 2, add 25.0.
13. " " " benzene " 0 1051 " " " " " " " " 2, " 65.0.

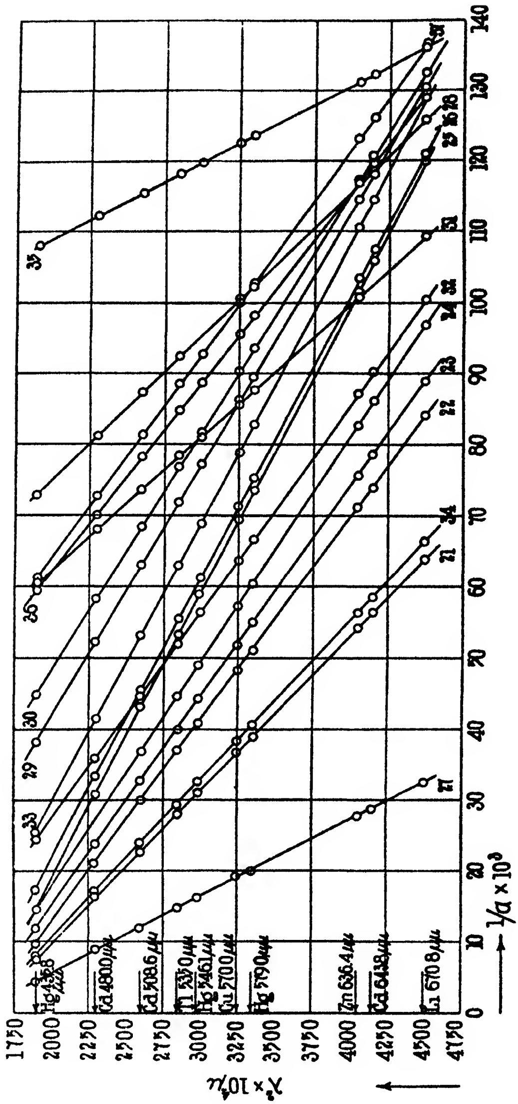

Hg 435.8 μμ
Cd 480.0 μμ
Cd 508.6 μμ
Tl 535.0 μμ
Hg 546.1 μμ
Cu 570.0 μμ
Hg 579.0 μμ
Zn 636.4 μμ
Cd 643.8 μμ
Li 670.8 μμ
1750 2000 2250 2500 2750 3000 3250 3500 3750 4000 4250 4500 4750
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140
$1/\alpha \times 10^3$
$\lambda^2 \times 10^4 \mu$
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

FIG. 2.

FIG. 2.

21.	β-Mannose	pentacetate in	chloroform,	concentration =	0 2870 gm. per cc.	Scale of $1/\alpha$, subtract 25.0.
22.	"	" "	"	"	0 4287 " " "	" " " add 40.0.
23.	"	" "	"	"	0.0211 " " "	" " " " 15 0, multiply by 10.
24.	"	" "	acetone	"	0 3476 " " "	" " " " 40 0.
25.	"	" "	"	"	0.1349 " " "	" " " " 55.0.
26.	"	" "	"	"	0.2779 " " "	" " " " 55.0.
27.	β-Glucose	" "	"	"	0 2360 " " "	" " " " 10.0.
28.	α-Glucose	" "	chloroform	"	0 3100 " " "	" " " divide by 2, subtract 2.0.
29.	"	" "	acetone	"	0.1641 " " "	" " " " " 2, add 10.0.
30.	"	" "	chloroform	"	0 0942 " " "	" " " " " 2, " 5 0.
31.	"	" "	acetone	"	0.3128 " " "	" " " " " 2, subtract 15.0.
32.	β-Galactose	" "	"	"	0 1860 " " "	" " " " " 2, add 32.5.
33.	"	" "	chloroform	"	0 1583 " " "	" " " " " 2, " 97 5.
34.	α-Glucose	" "	benzene	"	0.1462 " " "	" " " " " 2, " 14.5.
35.	β-Mannose	" "	"	"	0 0950 " " "	" " " subtract 90.0, multiply by 2.
36.	β-Glucose	" "	chloroform	"	0 2870 " " "	" " " " 25.0, " " 10.
37.	"	" "	benzene	"	0 0825 " " "	" " " " 25.0, " " 10.

As in the case previously reported, the experimental results presented here can be reproduced accurately by one term of Drude's equation:

$$[M] = \frac{K}{\lambda^2 - \lambda_0^2}$$

This fact is shown in Figs. 1 and 2 in which the reciprocals of the experimental rotations, not modified by any factors, are plotted as abscissas and the squares of the wave-lengths (expressed in μ) as ordinates. The scales of the graphs were chosen so that the smallest division corresponded to a change in rotation of less than 0.02°. It is readily seen that with the exception of a few occasional measurements, all the points lie on straight lines.

A more striking proof that the simple equation is sufficient to express the experimental points within the range of Li red and Hg violet is given in Table I. In Columns 3, 4, 7, and 8 are given the observed and calculated molecular rotations. In Columns 5 and 9 are given the differences between the two values expressed in per cent. It is obvious from these data, not only from the slight magnitude of the errors but also from the irregularities of the directions of these differences, that the assumption of the simple equation is rigorous.

In Table III are given the values of K at round concentrations. These values are not necessarily identical with those given in Table I. The latter values are dependent upon the experimental concentrations and therefore their accuracy is limited by the accuracy of the concentrations. The molecular rotations interpolated from a smooth curve give the average values and the values of K obtained from these values are necessarily more accurate, or at least more consistent.

In Table IV are given the differences between the values of K for the α and β forms of the pentacetate of glucose and of the pentacetate of mannose. Comparing the ratios

$$\frac{(K_\alpha - K_\beta)\text{ mannose pentacetate}}{(K_\alpha - K_\beta)\text{ glucose pentacetate}}$$

with the ratios

$$\frac{([M_\alpha] - [M_\beta])\text{ mannose pentacetate}}{([M_\alpha] - [M_\beta])\text{ glucose pentacetate}}$$

it is found that they have the same values. This agreement is to be expected when the course of the dispersión has the simple character capable of being expressed, as stated above, by a single term of Drude's equation.

The practical conclusion which follows from these observations is that for the solution of these problems in the case of the sugars which were studied by the optical method, the molecular rotations in any one monochromatic light are as significant as the dispersion constants.[3]

EXPERIMENTAL.

Procedure.

The apparatus employed, as well as the method used, was described in a recent publication.[2]

The green mercury line 5461, the violet 4359, and the yellow 5790, were obtained directly from a quartz mercury arc, the light from which was purified by means of a large, direct vision spectroscope. The rest of the lines used were obtained from a continuous spectrum, patches of which, having passed the spectroscope, were chosen so that the optical center of each patch had the desired wave-length. The optical centers were determined by means of a quartz test plate calibrated by the Bureau of Standards. The details of the procedure were described in an earlier publication.

Calculations.

The constant K was calculated in the usual way from Drude's formula. In Table III are given the values of this constant at round concentrations. The molecular rotations used for calculating the constants were taken from an earlier paper in which these values were read off from smooth curves drawn on large scales.

The constant λ_0^2 is very sensitive to slight variations in α, inasmuch as it is obtained by means of differences. The following modification of the simple equation was employed:

$$\lambda_0^2 = \frac{\alpha_1 \lambda_1^2 - \alpha_2 \lambda_2^2}{\alpha_1 - \alpha_2}$$

[3] We are indebted to Dr. L. W. Bass for verifying the tables.

where α is the experimental rotation as measured, not modified by any factors such as the concentrations or the lengths of the tubes. α_1 and α_2 were chosen so that the differences were as large as possible. As many combinations were employed as were found necessary to give each measurement equal weight. Often as many as twenty combinations were used.

The difference of α for Hg 5461 and Hg 4358 suggests itself as the most reliable combination. However, with the more concentrated solutions the measurements with the Hg violet line were not as accurate as with the others. This fact was indicated by a larger average of the mean deviation from the average.

TABLE I.

Comparison of Observed and Calculated Molecular Rotations for Different Wave-Lengths.

α-Mannose pentacetate in chloroform. $\lambda_0^2 = 0.0315\ \mu$.

Wave-length. μ	Concentration = 0 0430 gm per cc K = 6315				Concentration = 0 0775 gm per cc. K = 6878			
	α, 4 dm. tube	[M] observed	[M] calculated	Difference	α, 2 dm. tube	[M] observed	[M] calculated.	Difference
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	degrees			*per cent*	*degrees*			*per cent*
0 6708								
0 6438	7 65	177 6	177.9	+0.16	7.12	179 1	179 6	+0 22
0 6364	7 87	182 7	182 5	−0 10	7.34	184 7	184 2	−0 21
0 5790	9 67	224 5	224 3	−0 08	9 00	226 4	226.4	0
0 5700	10 00	232.1	232 3	+0 08	9.34	235 0	234 4	−0 25
0 5472	10 97	255 0	254 4	−0 23	10 21	256.9	256 8	−0 03
0 5461	11.07	257.0	255 5	−0 58	10 25	257 7	257 9	+0 07
0 5351	11 52	267 0	267 6	+0 22	10 75	270 0	270 0	0
0 5086	12 91	299 0	299 9	+0 30	12 04	302.9	302.7	−0 06
0.4800	14 71	341 0	342 5	+0.44	13 76	346 2	345.7	−0.14
0 4359	15 51	429 0	429 9	+0 20	17 15	431 5	433 9	+0 32

Wave-length μ	Concentration = 0 1566 gm per cc K = 6809				Concentration = 0 2410 gm per cc. K = 6574			
	α, 1 dm. tube	[M] observed.	[M] calculated	Difference.	α, 1 dm tube	[M] observed	[M] calculated	Difference
	degrees			*per cent*	*degrees*			*per cent*
0 6708								
0 6438	7 09	176 6	177 8	+0.67	10 63	172 0	171.7	−0 17
0 6364	7 31	182.1	182 3	+0.10	10 90	176 4	176 0	−0 22
0 5790	8 98	223 6	224.1	+0 22	13.37	216.4	216 4	0
0 5700	9 31	231 9	232.1	+0 08	13 82	223 6	224 1	+0 22
0 5472	10 20	254 0	254.1	+0.03	15 11	244 5	245 4	+0 36
0 5461	10.30	256.5	255.5	−0 46	15 30	247 6	246 5	−0 44
0 5351	10.76	268 0	267.3	−0 26	15 96	258 3	258 0	−0 11
0.5086	12 04	299 8	299 7	−0 03	17.90	289 7	289 4	−0 10
0 4800	13 78	343.2	342 3	−0 26	20.40	330 1	330 5	+0 12
0.4359	17 27	430.1	429 6	−0.11	25.52	413 0	414 8	+0.43

TABLE I—*Continued.*

ι-Mannose pentacetate in chloroform. $\lambda_0^2 = 0\ 0315\ \mu$.

Wave-length μ	Concentration = 0 3095 gm per cc K = 6413				Concentration = 0 4580 gm per cc. K = 6251			
	α, 1 dm tube	[M] observed	[M] calculated	Difference	α, 1 dm tube.	[M] observed	[M] calculated.	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708								
0 6438	13 22	166 6	167.4	+0 47	19.19	163 4	163 2	−0 12
0 6364	13 61	171 5	171.7	+0 11	19 67	167 5	167 4	−0 06
0 5790	16 81	211 8	211.1	−0 33	24.16	205.7	205.8	+0 04
0 5700	17.31	218 1	218 6	+0 22	24 97	212 6	213 0	+0 18
0 5472	19 05	240 0	239 4	−0.25	27 44	233 6	233 3	−0 12
0.5461	19 14	241 2	240 5	−0 29	27 60	235 0	234 4	−0 25
0 5351	19 97	251 6	251 7	+0 04	28 86	245.7	245 3	−0 16
0 5086	22 40	282 3	282 3	0	32 36	275 5	275 1	−0 15
0 4800	25 55	322 0	322 4	+0 12	36 85	313 8	314 3	+0 15
0 4359	32 01	403 3	404 6	+0 32	46 08	392 4	394 4	+0 51

Wave-length. μ	Concentration = 0 8033 gm per cc K = 6090			
	α, 1 dm tube.	[M] observed	[M] calculated.	Difference.
	degrees			*per cent*
0.6708				
0.6438	32.79	158.8	159.0	+0.12
0 6364	33.61	163.2	163.1	−0 06
0 5790	41 33	200 7	200.5	−0.09
0.5700	42 74	207.5	207.6	+0.04
0.5472	46.93	227.8	227.3	−0.22
0 5461	47 16	229.0	228.4	−0.26
0.5351	49 27	239.2	239 0	−0.09
0.5086	55.26	268.2	268.1	−0 03
0.4800	62 66	304.1	306.1	+0.65
0.4359	79.18	384.4	384.2	−0.06

TABLE I—*Continued.*

α-Mannose pentacetate in benzene. $\lambda_0^2 = 0.0326\ \mu$.

Wave-length. μ	Concentration = 0 1051 gm per cc. K = 7277			
	α, 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			*per cent*
0.6708				
0.6438	5.13	190.2	190.5	+0.09
0.6364	5.26	195.3	195.4	+0 04
0.5790	6.49	241.1	240.4	−0.25
0.5700	6 70	248 8	249 0	+0.03
0 5472				
0 5461	7.37	274.0	274.0	0
0 5351	7.72	286.7	286.8	+0 03
0 5086	8 66	321 8	321 8	0
0 4800	8 90	367.7	367 9	+0 04
0.4359	12 45	462 2	462 3	+0 01

α-Mannose pentacetate in acetone. $\lambda_0^2 = 0\ 0306\ \mu$.

Wave-length. μ	Concentration = 0 02306 gm per cc. K = 6046				Concentration = 0 0352 gm per cc. K = 6288			
	α, 4 dm tube.	[M] observed	[M] calculated.	Difference	α, 4 dm tube.	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0 6708	3.40	143.8	144 2	+0 28	5 24	150 1	149 9	−0 13
0.6438	3 74	157 8	157.5	−0.19	5.91	163 7	163 8	+0 06
0 6364	3 83	161 9	161.5	−0.27	6 07	168 1	168 0	−0 06
0 5790	4.72	199.4	198 4	−0 50	7 46	206 6	206 4	−0 09
0 5700	4 86	205.4	205 4	0	7 72	213 8	213 7	−0 04
0 5472								
0.5461	5 34	225 7	225 9	+0 09	8 48	234 9	235 0	+0.04
0.5351	5.58	235 9	236 4	+0.22	8 87	245 7	245 9	+0 08
0.5086	6.26	264 0	265.1	+0 43	9 94	275 0	275 6	+0 22
0.4800	7 12	301 0	302.6	+0 53	11 31	313 0	314 7	+0 54
0.4359	8 93	378 0	379 3	+0 34	14 24	394 0	394 5	+0 12

TABLE I—*Continued.*

α-Mannose pentacetate in acetone. $\lambda_0 = 0.0306\ \mu$.

Wave-length. μ	Concentration = 0 0744 gm per cc. K = 6287				Concentration = 0 2249 gm per cc. K = 6289			
	α, 4 dm tube.	[M] observed.	[M] calculated.	Difference	α, 2 dm tube.	[M] observed.	[M] calculated.	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708					17.22	149.1	149.9	+0 53
0.6438	12.11	163.5	163.7	+0.12	18.83	163.3	163.8	+0.31
0.6364	12.44	167 6	167.9	+0.18	19.30	167 3	168.0	+0.41
0.5790	15.31	206 7	206 4	−0.14	23.77	206 0	206 4	+0.19
0.5700	15.83	213 8	213.5	−0 14	24.60	213 3	213 7	+0.18
0.5472	17.34	234.1	233 9	−0 08				
0.5461	17.40	234.9	234.9	0	27.10	235 0	235.0	0
0.5351	18.25	246 4	245 9	−0 20	28.38	246 1	246.0	−0.04
0.5086	20 45	276 0	275 6	−0 10	31 75	275.2	275 7	+0 18
0.4800	23 30	314.0	314 7	+0.21	36 56	317.0	314.8	−0 68
0.4359	29 05	392 0	394 4	+0.60	45 13	390.2	394 5	+1 00

Wave-length. μ	Concentration = 0 2674 gm per cc. K = 6263				Concentration = 0.3329 gm per cc. K = 6148			
	α, 1 dm. tube.	[M] observed	[M] calculated.	Difference.	α, 1 dm. tube	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708	10.11	148.5	149 3	+0.53	12 50	146 4	146.6	+0 13
0 6438	11 08	162 6	163.1	+0.30	13.66	160.0	160.1	+0 06
0.6364	11 36	166 7	167.3	+0.35	14.03	164.4	164 2	−0 12
0.5790	14.01	205 6	205 5	−0 04	17.25	202.1	201 8	−0.14
0.5700	14 50	212 8	212 8	0	17.84	209.0	208.9	−0 05
0 5472					19.54	228.9	228.7	−0 08
0.5461	15 96	234 3	234 0	−0.12	19 64	230 1	229 7	−0 17
0.5351	16 67	244.7	244 9	+0.08	20.55	240.7	240 5	−0 08
0.5086	18 87	277.0	274 6	−0.87	23 03	269.8	269.5	−0.10
0 4800	21 46	315 0	313 4	−0.51	26 27	307 8	307 7	−0 03
0.4359	26 68	391.6	392 9	+0 33	32.70	383 1	385.7	+0.67

TABLE I—*Continued.*

α-Mannose pentacetate in acetone. $\lambda_0^2 = 0.0306\ \mu$.

Wave-length μ	Concentration = 0.4998 gm per cc. K = 6005				Concentration = 0 6871 gm. per cc. K = 5915			
	α, 1 dm. tube.	[M] observed.	[M] calculated	Difference.	α, 1 dm. tube	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708	18 35	143.2	143 2	0	24.80	140.8	141.0	+0.15
0 6438	19.99	156 0	156 4	+0.25	27.15	154 1	154 1	0
0 6364	20 53	160.2	160 4	+0.12	27 80	157.8	158 0	+0 12
0.5790	25.26	197 5	197 1	−0.20	34 31	194.7	194 1	−0 30
0.5700	26.15	204 0	204.0	0	35 41	201 0	201 0	0
0 5472								
0 5461	28 76	224.4	224 4	0	38 99	221 2	221 0	−0.09
0 5351	30 12	235.0	234 8	−0 08	40.75	231.3	231.3	0
0.5086	33.77	263 5	263 3	−0.08	45.67	259 2	259 3	+0.04
0 4800	38 56	300 9	300 6	−0 10	52 10	295 7	296 0	+0.01
0 4359	48 40	377 7	376 7	−0 21				

β-Mannose pentacetate in acetone. $\lambda_0 = 0\ 0185\ \mu$.

Wave-length. μ	Concentration = 0 1349 gm per cc. K = 3571				Concentration = 0 2779 gm per cc. K = 3548			
	α, 2 dm tube	[M] observed.	[M] calculated.	Difference.	α, 1 dm. tube	[M] observed	[M] calculated.	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708	−5.72	−82.70	−82.76	+0.07	−5 84	−82 01	−82 22	+0.25
0.6438	−6.22	−89 90	−90.18	+0.31	−6 36	−89 25	−89 59	+0.37
0.6364	−6 39	−92.4	−92 40	0	−6 52	−91.50	−91.80	+0.32
0.5790	−7 81	−113.0	−112.7	−0 23	−7.99	−112 1	−112 0	−0 08
0 5700	−8 05	−116 4	−116 5	+0.08	−8 25	−115 8	−115.8	0
0 5472								
0.5461	−8.83	−127.6	−127 6	0	−9 05	−126 9	−126 8	−0.08
0.5351	−9.22	−133 3	−133.3	0	−9 45	−132 6	−132 5	−0.07
0.5086	−10.31	−149 0	−148.7	−0 20	−10 55	−148.0	−147.7	−0.20
0.4800	−11.65	−168.4	−168.5	+0.06	−11 94	−167 6	−167.4	−0 11
0.4359					−14 80	−207 7	−206.9	−0 38

TABLE I—*Continued.*

β-Mannose pentacetate in acetone. $\lambda_0^2 = 0.0185\ \mu$.

Wave-length μ	Concentration = 0.3476 gm per cc. K = 3531			
	α, 1 dm tube	[M] observed.	[M] calculated	Difference.
	degrees			*per cent*
0.6708	−7.30	−82.0	−81 8	−0 24
0.6438	−7.95	−89 4	−89 2	−0.22
0.6364	−8 14	−91.5	−91 4	−0.12
0.5790	−9 93	−111.6	−111.5	−0.08
0 5700	−10 24	−115 1	−115.2	+0.08
0.5472				
0.5461	−11 22	−126 1	−126 1	0
0 5351	−11 73	−131.8	−131 8	0
0 5086	−13 06	−146 8	−147 0	+0 13
0 4800	−14 79	−166 3	−166 6	+0 18
0 4359	−18 28	−205 5	−205 9	+0 19

β-Mannose pentacetate in benzene. $\lambda_0^2 = 0\ 0239\ \mu$.

gth	Concentration = 0 0950 gm per cc K = 3797			
	α, 1 dm. tube	[M] observed	[M] calculated	Difference.
	degrees			*per cent*
0 6708	−2.17	−89 05	−89.11	+0 06
0 6438	−2.29	−97 05	−97.21	+0 16
0 6364	−2 43	−99 59	−99.63	+0 03
0.5790	−2 98	−122 2	−121.9	−0 24
0.5700	−3 07	−125.9	−126.1	+0.15
0 5472				
0.5461	−3 52	−138 3	−138.4	+0 07
0 5351	−3 94	−144 6	−144 7	+0 06
0 5086	−4 48	−161 6	−161 7	+0.06
0.4800	−5.58	−184.1	−183 9	−0.10
0 4359	−6 94	−229.1	−228 6	−0 22

TABLE I—*Continued.*

β-Mannose pentacetate in chloroform. $\lambda_0^2 = 0.0159\mu$.

Wave-length. μ	Concentration = 0 0211 gm per cc K = 3106				Concentration = 0 2870 gm per cc K = 3142			
	α, 4 dm. tube.	[M] observed.	[M] calculated	Difference	α, 2 dm tube	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0 6708	−1 55	−71 60	−71 57	−0 04	−10.65	−72 30	−72 40	+0 13
0.6438	−1 69	−78.00	−77 94	−0 07	−11 58	−78 70	−78 85	+0 19
0 6364	−1 73	−79 90	−79 85	−0 06	−11 87	−80 60	−80 77	+0 21
0 5790	−2 11	−97 40	−97 28	−0 12	−14.49	−98 40	−98 40	0
0.5700	−2.17	−100 2	−100 5	+0 29	−14 99	−101 8	−101 7	−0 09
0.5472								
0 5461	−2 37	−109.4	−110 1	+0 63	−16 38	−111 3	−111 3	0
0.5351	−2 49	−115 0	−114 9	−0 08	−17 11	−116 2	−116 2	0
0 5086	−2 77	−127 9	−128 0	+0 07	−19 09	−129 7	−129 5	−0 15
0 4800	−3 15	−145 4	−144.9	−0 35	−21 54	−146 3	−146 5	+0 13
0 4359	−3 86	−178 2	−178 5	+0 16	−26 63	−180 9	−180 6	−0 16

Wave-length. μ	Concentration = 0 1287 gm per cc. K = 3178			
	α, 1 dm. tube	[M] observed	[M] calculated	Difference
	degrees			*per cent*
0.6708	−8.05	−73.23	−73 23	0
0.6438	−8.77	−79.78	−79.75	−0.03
0 6364	−8 97	−81 60	−81 70	+0.12
0.5700	−10 95	−99.61	−99.53	−0 08
0 5700	−11.33	−103.1	−102.9	−0.19
0.5472				
0.5461	−12 35	−112 4	−112 6	+0.17
0.5351	−12 94	−117.7	−117 6	−0 08
0.5086	−14 40	−131.0	−131.0	0
0.4800	−16 29	−148 2	−148.2	0
0.4359	−20 06	−182.5	−182 6	+0.06

TABLE I—*Continued.*

α-Glucose pentacetate in benzene. $\lambda_0^2 = 0.0258\mu$.

Wave-length. μ	Concentration = 0 1462 gm per cc K = 11880			
	α, 2 dm. tube	[M] observed	[M] calculated.	Difference.
	degrees			*per cent*
0 6708	20 99	140.0	140.0	0
0 6438	22.96	152.8	152.8	0
0 6364	23 50	156.7	156.6	−0.03
0 5790	28 86	192 4	191 9	−0.25
0.5700	29.76	198 4	198 7	+0.12
0 5472				
0 5461	32 68	217.9	218 0	+0.05
0.5351	34.15	227 7	228 0	+0 10
0.5086	38.24	255 0	255.0	0
0.4800	43 48	290 0	290 3	+0 10
0 4359	54 27	362 0	361 8	−0 06

α-Glucose pentacetate in chloroform. $\lambda_0^2 = 0\ 0260\mu$.

Wave-length. μ	Concentration = 0 0[illegible]42 gm per cc. K = 12360				Concentration = 0 3100 gm. per cc K = 12430			
	α, 2 dm tube	[M] observed	[M] calculated	Difference.	α, 1 dm. tube	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0.6708	14.08	291.2	291 5	+0.10	23.24	292 4	293 1	+0 20
0 6438	15 33	317 3	318.1	+0.22	25.38	319 3	319.9	+0 18
0 6364	15 75	326 0	326.1	+0.03	26 06	327.8	328.0	+0 06
0 5790	19 31	399.7	399 6	−0 02	31.91	401.4	401 9	+0.12
0.5700	19 96	413.2	413.5	+0 07	33.05	415 8	415 9	+0 02
0 5472								
0 5461	21.98	450 0	454 1	+0 90	36.35	457.4	456.6	−0 17
0 5351	22.97	475.5	474 8	−0.14	37 99	477.9	477 5	−0.08
0.5086	25 69	532.0	531 2	−0 15	42 52	534 9	534 2	−0 11
0.4800	29 17	604 0	604.7	+0.11	48 36	608 4	608 1	−0 04
0.4359	36.24	750.0	753.7	+0.49	60 21	757.5	757.9	+0.05

TABLE I—*Continued.*

α-Glucose pentacetate in acetone. $\lambda_0^2 = 0.0265\mu$.

Wave-length, μ	Concentration = 0 1641 gm. per cc. K = 13360				Concentration = 0 3128 gm. per cc K = 13320			
	α, 1 dm tube	[M] observed	[M] calculated.	Difference.	α, 1 dm tube.	[M] observed	[M] calculated	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0 6708	13 27	315 4	315 5	+0 03	25 16	313 7	314 5	+0.25
0.6438	14 48	344 1	344 3	+0 04	27.53	343 3	343 3	0
0.6364	14 89	353 9	353 0	−0 25	28.23	352 0	351 9	−0 03
0 5790	18 20	432.5	432.6	+0 02	34.60	431 4	431 3	−0 02
0.5700	18 83	447 5	447 7	+0 04	35.83	446.7	446 4	−0 07
0.5472								
0.5461	20 68	491 5	491 7	+0 04	39 34	490 5	490.3	−0 04
0.5351	21 66	514 8	514 2	−0 11	41 18	513 4	512 7	−0 11
0.5086	24 22	575 6	575 4	−0 03	46 07	574 4	573 6	−0 14
0 4800	27 60	655.9	655 2	−0.10	52 44	653 8	653 3	−0 08
0.4359	34 30	815 2	817.1	+0 23	65 27	813.8	814 8	+0 12

α-Glucose pentacetate in pyridine. $\lambda_0 = 0.0231\mu$.

Wave-length μ	Concentration = 0 1998 gm per cc K = 8689				Concentration = 0 3004 gm. per cc K = 8890			
	α, 1 dm. tube.	[M] observed	[M] calculated.	Difference	α, 1 dm tube	[M] observed	[M] calculated.	Difference
	degrees			*per cent*	*degrees*			*per cent*
0.6708	10 41	203 2	203 5	+0 14	15 98	207 5	208 2	+0 33
0.6438	11 33	221.2	222 0	+0 36	17 45	226 6	227.1	+0 22
0.6364	11.65	227 4	227 5	+0 04	17 87	234 7	232 8	−0 81
0.5790	14 26	278 4	278.3	−0 04	21 93	284.7	284.8	+0 04
0.5700	14 74	287 8	287 9	+0 03	22 66	294 2	294.6	+0.13
0.5472								
0.5461	16 18	315 9	316 0	+0 03	24 89	323 2	323 3	+0 03
0.5351	16 91	330 2	330 1	−0 03	26 02	337 9	337.8	−0 02
0.5086	18 90	368 9	368 8	−0 02	29 13	378 2	377.3	−0 24
0.4800	21 50	419 7	419.2	−0 12	32 96	428 0	428 8	+0.18
0.4359	26.72	521 6	520 6	−0.18	40 98	532 1	532.6	+0 09

TABLE I—*Continued.*

β-Glucose pentacetate.

In benzene. $\lambda_0^2 = 0.0663\mu$. In chloroform. $\lambda_0^2 = 0.0595\mu$.

Wave-length μ	Concentration = 0 0825 gm per cc K = 405 7				Concentration = 0 2870 gm per cc. K = 511.8			
	α, 4 dm tube	[M] observed	[M] calculated.	Difference	α, 1 dm. tube.	[M] observed	[M] calculated.	Difference.
	degrees			*per cent*	*degrees*			*per cent*
0 6708	0 89	10 57	10 57	0	0 96	13 10	13 11	+0 07
0 6438	0 98	11 64	11 65	+0 08	1 06	14 41	14 41	0
0 6364	1 01	11 97	11 98	+0 08	1 09	14 80	14 81	+0 06
0 5790	1 28	15 13	15 08	−0 33	1 37	18 60	18 56	−0 21
0 5700	1 33	15 69	15 69	0	1 42	19 27	19.28	+0 05
0 5472								
0 5461	1 48	17 50	17 50	0	1 58	21 43	21 44	+0 04
0 5351	1 56	18 44	18 44	0	1 66	22 57	22 57	0
0 5086	1 78	21 09	21 09	0	1 89	25 69	25 69	0
0 4800	2 09	24 67	24 72	+0 20	2 20	29 93	29 95	+0 06
0 4359	2 77	32 75	32 80	+0 15	2 89	39 22	39 22	0

β-Glucose pentacetate in acetone. $\lambda_0^2 = 0\ 0569\mu$.

Wave-length μ	Concentration = 0 2360 gm per cc K = 761 7			
	α, 2 dm. tube	[M] observed.	[M] calculated.	Difference
	degrees			*per cent*
0.6708	2.34	19 38	19 38	0
0 6438	2.58	21 31	21 30	−0.04
0 6364	2 65	21.89	21 88	−0.04
0 5790	3.35	27 38	27.36	−0.09
0 5700	3.43	28.34	28 42	+0.28
0 5472				
0 5461	3.82	31 57	31 70	+0.41
0 5351	4.02	33 21	33 20	−0 03
0.5086	4.57	37.77	37 75	−0.05
0 4800	5 32	43 92	43 90	−0.04
0.4359	6 94	57.30	57 23	−0.12

TABLE I—*Concluded.*

β-Galactose pentacetate.

In chloroform. $\lambda_0^2 = 0.0384\mu$. In acetone. $\lambda_0^2 = 0.0365\mu$.

Wave-length μ	Concentration = 0.1583 gm per cc K = 3102				Concentration = 0.1860 gm per cc K = 3609			
	α, 2 dm tube	[M] observed	[M] calculated	Difference	α, 2 dm tube	[M] observed	[M] calculated	Difference
	degrees			*per cent*	*degrees*			*per cent*
0.6708	6.10	75.1	75.3	+0.26	8.29	86.9	87.2	+0.34
0.6438	6.69	82.4	82.5	+0.12	9.08	95.2	96.1	+0.93
0.6364	6.86	84.5	84.6	+0.11	9.93	97.8	97.9	+0.10
0.5790	8.49	104.5	104.5	0	11.52	120.7	120.7	0
0.5700	8.81	108.5	108.3	−0.18	11.96	125.4	125.1	−0.23
0.5472								
0.5461	9.70	119.4	119.4	0	13.11	137.4	137.9	+0.36
0.5351	10.15	125.0	125.2	+0.15	13.82	144.9	144.4	−0.34
0.5086	11.46	141.1	140.8	−0.21	15.55	163.0	162.4	−0.36
0.4800	13.17	162.2	161.6	−0.37	17.85	187.2	186.1	−0.57
0.4359	16.57	204.1	204.6	+0.24	22.36	234.4	235.1	+0.29

TABLE II.

Values of the Constants λ and K in Table I.

Substance	Solvent	Concentration in gm. per cc. $\times 10^2$.	λ_0^2	$K \times 10^{-2}$
			μ	
α-Mannose pent-acetate.	Chloroform.	4.300	0 0315	63.15
		7.750		68.78
		15.66		68.09
		24 10		65.74
		30.95		64.13
		45.80		62.51
		80.33		60.90
	Benzene.	10 51	0 0326	72 77
	Acetone.	2 306	0 0306	60.46
		3.520		62.88
		7.440		62.87
		22.49		62 89
		26.74		62.63
		33 29		61.48
		49 98		60.05
		68.71		59 15
β-Mannose pent-acetate.	Chloroform.	2 110	0 0159	31 06
		28 70		31.42
		42.87		31 78
	Acetone.	13.49	0 0185	35.71
		27.79		35.48
		34.76		35.31
	Benzene.	9 500	0 0239	37 97
α-Glucose pent-acetate.	Chloroform.	9 420	0 0260	123.6
		31.00		124.3
	Acetone.	16.41	0.0265	133.6
		31.28		133.2
	Benzene.	14.62	0.0258	118 8
	Pyridine.	19 98	0 0231	86.89
		30 04		88.90
β-Glucose pent-acetate.	Chloroform.	28 70	0 0595	5.118
	Acetone.	23 60	0 0569	7.617
	Benzene.	8 250	0 0663	4 057
β-Galactose pent-acetate.	Chloroform.	15 83	0.0384	31.02
	Acetone.	18 60	0 0365	36 09

TABLE III.

Values of K at Round Concentrations.

$K \times 10^{-2}$

Concentration in gm. per cc $\times 10^3$	α-Mannose pentacetate.			β-Mannose pentacetate.		
	Chloroform.	Acetone.	Benzene	Chloroform.	Acetone	Benzene.
2.00	66 67	58 34	72 51	31 04	34 99	38 40
4 00	65 07	61 01	72 77	31 04	35.17	38 40
6 00	67.47	62 08	72.77	31 04	35.41	38 57
8 00	67 74	62 35	72.77	31 04	35 69	38 74
10 0	67.74	62.62	73 31	31 04	35 83	37 58
12 0	68 01	62 62	72 51	31 04	35 83	37 30
14 0	68.01	62.62	72 77	31 04	35.83	36.61
16 0	68 11	62 62	72.51	31 04	35 83	36 94
18 0	67 55	62.75	72 06	31 04	35.83	36 21
20 0	67 34	62 78	72 24	31 04	35.83	36 21
22 0	66 56	62 75	71 71	31 04	35.83	36.12
24 0	65 93	62 78	71 31	31 32	35.55	35 93
25 0	65.39	62.78	71 02	31 32	35 55	35 84
30.0	64.57	62 06	70.23	31 32	35 55	35 66
35.0	63.77	61 36		31 61	35 27	
40 0	63 07	60 82		31 61	35 27	
45 0	64 19	60 42		31 61	35.27	
50.0	62 33	60 08		31 61		
60 0	61 79	59.51		31 61		
70.0	61 39					
80 0	60 97					

Concentration in gm per cc $\times 10^3$	α-Glucose pentacetate.			β-Glucose pentacetate		
	Chloroform.	Acetone	Benzene	Chloroform	Acetone.	Benzene
2.00	122 1	132 5	118 4	5 59	7.02	4 29
4 00	123 9	132 5	118 4	5 23	7 06	4 29
6.00	124 4	132 5	118.4	5 16	7 02	4 31
8.00	124 3	132 8	118 7	5 04	7.05	4 34
10 0	123 9	132 9	118 5	5 04	7.02	4 34
12 0	123 9	133 0	118 4	5 01	7 02	4 34
14 0	123 6	133 1	118.4	4 96	7 16	4 29
16.0	123 9	133 4	118 7	4 96	7 28	
18 0	124.1	133.3	119.5	4.96	7.40	
20 0	124 2	133 2	120 0	4.94	7.80	
22 0	124 5	133.1	120 4	4.92	7 57	
24 0	124.7	133 1	120 7	4 92	7.58	
26 0	124 9	133.2	120.9	4 96	7.64	
28 0	125 1	133.1	121 0	5.06	7.68	
30 0	125 0	133 0		5 25	7.71	
35 0	125 6	133.0		5.37		
39 0	126 0	133 0		5.44		
44 0	126 2			5.61		

TABLE IV.

Differences in Values of K for α- and β-Pentacetates at the Same Concentration.

Concentration in gm per cc $\times 10^2$	Mannose pentacetate. $K_\alpha - K_\beta$			Glucose pentacetate. $K_\alpha - K_\beta$		
	Chloroform.	Acetone	Benzene.	Chloroform.	Acetone.	Benzene
2 00	97.71	93 33	110 91	115 5	125.5	114 1
4 00	96 11	96 18	111.17	118 7	125.4	114 1
6.00	98 51	97 49	111.34	119 3	125.5	114.1
8 00	98 78	98.04	111 51	119 3	125.8	114.4
10.0	98.78	98 45	110.89	118 9	125 9	114.2
12 0	99 05	98 45	109.81	118 9	126.0	114.1
14 0	99 05	98 45	109 38	118 6	126 0	114 1
16 0	99 15	98 45	109 45	118.9	126.1	
18.0	98 59	98 57	108 27	119 1	125 9	
20 0	98 38	98 61	108 45	119 2	125.7	
22.0	97.60	98.58	107 83	119.5	125.5	
24.0	97 25	98 33	107 24	119.7	125 5	
25 0	96 71	98 33	106 86	119 9	125 6	
30.0	95 89	97 61	105 89	119 8	125 3	
35.0	95 35	96 63		120 3		
40 0	94 68	96 09		120 6		
45 0	95.80	95 69		120 6		
50 0	93 94					
60.0	93 40					
70 0						
80.0						

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1927,
Vol. lxxiv, No. 2, pp. 343–350.]

CONFIGURATIONAL RELATIONSHIPS OF 2-HYDROXY-BUTYRIC AND LACTIC ACIDS.

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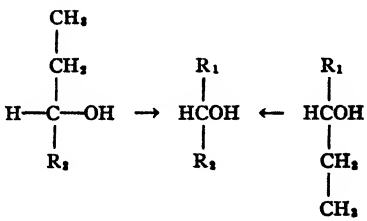
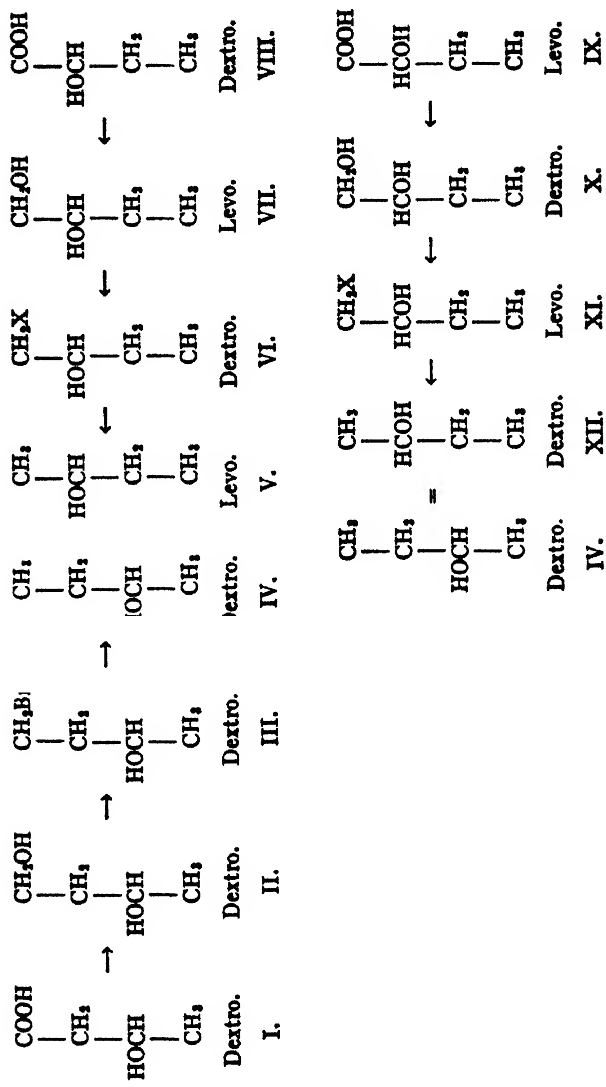
(Received for publication, June 6, 1927.)

The correlation of the configurations of the individual α-hydroxy acids has not yet been established by direct chemical methods. Indirect methods were suggested by Clough, by Hudson, and by Levene. It is therefore important to test by the direct method the conclusions reached by the indirect methods. The plan for the direct chemical method is the following. Dextro-lactic acid was shown to be configurationally related to dextro-3-hydroxybutyric acid (β-hydroxybutyric acid) and to dextro-1,3-dihydroxybutane and the latter in its turn has been correlated with dextro-methylethyl carbinol. From the figures on p. 240 it is seen clearly that 2-hydroxybutyric acid having the hydroxyl on the same side as dextro-3-hydroxybutyric acid should lead to a levo-methylethyl carbinol; whereas 2-hydroxybutyric acid, configurationally related to levo-3-hydroxybutyric acid, should lead to dextro-ethylmethyl carbinol. To make the reasoning more comprehensible, one may represent the relationship of dextro- and levo-ethylmethyl carbinols to dextro-lactic acid in the following way.



XIII. XIV. XV.

It is evident that when R_1 (in XV) is made to be the same group as R_2 (in XIII) then (XIII) will be enantiomorphous to (XV).


Dextro. I.
Dextro. II.
Dextro. III.
Dextro. IV.
Levo. V.
Dextro. VI.
Levo. VII.
Dextro. VIII.
Levo. IX.
Dextro. X.
Levo. XI.
Dextro. XII.
Dextro. IV.

The plan of the work leading to the correlation of the configuration of 2-hydroxybutyric acid with lactic acid and with methylethyl carbinol was analogous to that which led to the correlation of 3-hydroxybutyric acid with lactic acid and with methylethyl carbinol.

The transformation of dextro-2-hydroxybutyric acid into levo-1,2-dihydroxybutane may be regarded as the first step in the work. The second step consisted of the preparation of 1,2-dihydroxybutane starting from chloromethylethyl ketone, the conversion of this compound into hydroxymethylethyl ketone, and the reduction of the latter by fermentation to dextro-1,2-dihydroxybutane. The glycol was converted into the levo-chlorohydrin, which was finally reduced to dextro-ethylmethyl carbinol. Hence, levo-1,2-dihydroxybutane derived from dextro-2-hydroxybutyric acid will yield levo-ethylmethyl carbinol. This result, as pointed out above, is to be expected if dextro-2-hydroxybutyric acid has the same configuration as dextro-lactic acid; namely, if it belongs to the *l* series of hydroxy acids.

The opposite conclusion reached by Clough was based on an error, inasmuch as the direction of the rotation of the free acid was not taken into consideration by this author. Clough designated as levo-hydroxybutyric acid that acid the salt of which was levorotatory. From the data presented in this paper, it is seen that the levorotatory ammonium salt of 2-hydroxybutyric acid leads to a dextrorotatory free acid.

On the other hand, the rule of Levene for determining the configuration of 2-hydroxy acids on the basis of the sign of the difference of the rotation of the undissociated acid and of its ion is fully substantiated by the method developed in the present communication.

EXPERIMENTAL.

Levo- and Dextro-2-Hydroxybutyric Acids.—The inactive acid was obtained from 2-bromobutyric acid by the procedure described by Bischoff and Walden.[1]

An aqueous solution of the free acid was neutralized with morphine; the solution was heated on the steam bath for half an hour and then filtered. The filtrate was concentrated under reduced pressure to a

[1] Bischoff, C. A., and Walden, P., *Ann. Chem.*, 1894, cclxxix, 102.

thick syrup. When this was left in the ice box overnight, part of it crystallized. The crystals were removed on a Buchner funnel and then twice recrystallized from 50 per cent alcohol.

The morphine salt was dissolved in water and decomposed by the addition of a slight excess of ammonia. The morphine was removed by filtration and the filtrate converted into the barium salt in the usual way. In water the barium salt had the following rotation.

$$[\alpha]_D^{24} = \frac{+0.43° \times 100}{1 \times 5.6} = +7.7°.$$

A barium salt prepared from the acid obtained on decomposition of the mother liquors in the above resolution yielded the following rotation.

$$[\alpha]_D^{22} = \frac{-0.56° \times 100}{2 \times 5.0} = -5.6°.$$

The free acid was liberated from the second salt in the following manner. 2.0 gm. of barium salt ($[\alpha]_D^{22} = -5.6°$) were dissolved in cold water and 5.0 cc. of 2.32 N hydrochloric acid added. The volume was made up to 10 cc. and the rotation taken immediately.

$$[\alpha]_D^{22} = \frac{+0.54° \times 100}{2 \times 12} = +2.3°.$$

Ethyl-Levo-n-2-Hydroxybutyrate.—100 gm. of thoroughly dried barium 2-hydroxybutyrate ($[\alpha]_D^{22} = -5.6°$) were suspended in 250 cc. of absolute alcohol and a solution of 34 gm. of concentrated sulfuric acid in 100 cc. of absolute alcohol was slowly dropped in, the mixture being vigorously stirred with a mechanical stirrer. The mixture was heated under a reflux condenser on a boiling water bath for 8 hours. After cooling, dry ether was added, the excess sulfuric acid was neutralized with solid potassium carbonate, and the solution filtered from salts. It was then dried over anhydrous sodium sulfate, the solvent removed, and the ester was distilled under reduced pressure. It boiled at 64–66°C., p = 20 mm., and rotated without solvent as follows:

$$[\alpha]_D^{22} = \frac{-3.75° \times 1}{1 \times 0.978} = -3.83°.$$

It analyzed as follows:

0.1134 gm. substance: 0.2264 gm. CO_2 and 0.0938 gm. H_2O.

$C_6H_{12}O_3$.	Calculated.	C 54.40, H 9.16.
	Found.	" 54.44, " 9.25.

An ester prepared from a barium salt ($[\alpha]_D^{24} = +7.7°$) had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 8.40° \times 1}{1 \times 0.978} = + 8.59°.$$

Levo-1,2-Dihydroxybutane.—Ethyl-2-hydroxybutyrate ($[\alpha]_D^{22} = -3.8°$) was reduced with sodium and glacial acetic acid in the apparatus described by Levene and Allen.[2] The procedure was the same as that described previously for the reduction of other hydroxy acids. The glycol boiled at 94–96°C., p = 12 mm.

It analyzed as follows:

0.0936 gm. substance: 0 1827 gm. CO_2 and 0.0916 gm. H_2O.

$C_4H_{10}O_2$.	Calculated.	C 53.27, H 11 20.
	Found.	" 53 22, " 10 95.

27 gm. of ethyl-2-hydroxybutyrate yielded 2 gm. of the glycol.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{- 0.63° \times 100}{2 \times 4.25} = - 7.4°.$$

Di-(Phenylurethane) of Levo-1,2-Dihydroxybutane.—The urethane was prepared in the usual way. Recrystallized from dilute alcohol several times, it melted at 121–123°C. and analyzed as follows:

0.1000 gm. substance· 6 33 cc. 1N HCl.

$C_{18}H_{20}N_2O_4$.	Calculated.	N 8.54.
	Found.	" 8.86.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{- 2.28° \times 100}{2 \times 5.74} = - 19.8°.$$

[2] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

Preparation of Optically Active 1, 2-Dihydroxybutane from Methylethyl Ketone.

Chloromethylethyl Ketone (*1-Chlorobutanone-2*).—This product was obtained by the chlorination of methylethyl ketone, the procedure employed being essentially the same as that described by Kling.[3] The variation introduced was that twice as much marble was used. After the reaction mixture had been dried over calcium chloride it was distilled under reduced pressure. It was then subjected to repeated fractional distillations with an efficient fractionating column. A fraction which boiled constantly at 138.8–139.2°C. (corrected), p = 755 mm., was obtained.

It analyzed as follows:

0.1228 gm. substance 0 1694 gm. AgCl.

C_4H_7OCl.	Calculated	Cl	33 33.
	Found.	"	34 12.

Hydroxymethylethyl Ketone (*Butanol-*(*1*)*-one-*(*2*)).—100 gm. of chloromethylethyl ketone (b.p. = 138 8–139 2°C.), 160 gm. of dried potassium formate, and 160 cc. of dry methyl alcohol were heated under a reflux condenser on a water bath overnight.

The reaction mixture was cooled, dry ether was added, and the solution was then filtered. The solvent was removed and the hydroxy ketone distilled under reduced pressure. It was then redistilled under reduced pressure employing a flask provided with a Vigreux column. The fraction, which boiled at 50.5–51°C., p = 14 mm., was collected and used for reduction to the glycol.

Dextro-1, 2-Dihydroxybutane.—This glycol was obtained by the reduction of hydroxymethylethyl ketone with fermenting yeast.

To an actively fermenting mixture of 45 gm. of cane sugar, 450 gm. of bakers' yeast, and 4500 cc. of water were added 45 gm. of hydroxymethylethyl ketone. The reaction mixture was allowed to stand 6 days and then worked up in the usual way.[4] The glycol thus obtained on redistillation from a flask provided with a Vigreux column boiled at 75.0–75.5°C., p = 1 to 1.5 mm. Another lot distilled at 91–91.5°C., p = 13 mm.

[3] Kling, A., *Compt. rend. Acad.*, 1905, cxl, 312.

[4] Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96. Farber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

It analyzed as follows:

0.1076 gm. substance:	0.2104 gm. CO_2 and	0.1050 gm. H_2O.	
0.1238 " "	0.2397 " " "	0.1252 " "	
$C_4H_{10}O_2$.	Calculated.	C 53.27, H 11 20.	
	Found.	" 53.32, " 10 92.	
		" 52.79, " 11.31.	

In absolute alcohol it had the following rotation.

$$\frac{+\ 2.00^\circ \times 100}{1 \times 16.1} = +\ 12.4^\circ.$$

Di-(Phenylurethane) of Dextro-1, 2-Dihydroxybutane.—This substance was prepared in the usual way. Several recrystallizations from dilute alcohol gave a product which melted at 125–127°C. and analyzed as follows:

0 1000 gm. substance: 5 90 cc. 1 N HCl.		
$C_{18}H_{20}N_2O_4$.	Calculated.	N 8 54.
	Found.	" 8.26.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+\ 1.08^\circ \times 100}{1 \times 4.55} = +\ 23.7^\circ.$$

Conversion of Optically Active 1, 2-Dihydroxybutane into Secondary Butyl Alcohol.

Levo-1-Bromo-2-Hydroxybutane.—This bromohydrin was prepared from dextro-1, 2-dihydroxybutane ($[\alpha]_D^{22} = +12.4°$) in the usual way.[5] It boiled at 61–63°C., p = 12 mm. The optical rotation in a 1 dm. tube without solvent was $\alpha_D^{22} = -11.8°$, t = 22°C.

Dextro-Ethylmethyl Carbinol.—The bromohydrin obtained in the above experiment was reduced in alkaline solution with hydrogen in the presence of colloidal palladium by the procedure previously described.[5] After drying over anhydrous sodium sulfate, the ether was removed with the aid of a Vigreux column. The alcohol was then

[5] Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 465. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 591.

distilled and a fraction which boiled at 98–99°C. was collected. In alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{+1.15^\circ \times 100}{1 \times 8.82} = +13.0^\circ.$$

It was converted into the phenylurethane in the usual manner. This analyzed as follows:

0.1000 gm. substance: 5 00 cc. 1 N HCl.
$C_{11}H_{13}O_3N$. Calculated. N 7.26.
Found. " 7.00.

It melted at 61–63°C. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{+1.08^\circ \times 100}{1 \times 4.08} = +26.5^\circ.$$

[Reprinted from COMPTES RENDUS DES SÉANCES DE LA SOCIÉTÉ DE BIOLOGIE, May 6, 1927, Vol. xcvi, No. 14, pp. 1119–1121]

LA MALIGNITÉ DES FIBROBLASTES SARCOMATEUX DU RAT EN CULTURE PURE.

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Les propriétés principales de l'élément malin du sarcome fuso-cellulaire de la Poule et des monocytes du sang transformés *in vitro* en cellules sarcomateuses par le virus de Rous ont été décrites dans une note précédente (1). Ce type de cellule maligne se distingue du macrophage normal, surtout par sa fragilité et sa faculté de digérer la fibrine du coagulum. On ne peut donc pas conserver pendant longtemps une culture pure de macrophages sarcomateux sans y ajouter souvent du plasma frais et des monocytes normaux, ou bien, comme le fait Fischer, des fragments de tissu musculaire. Il était intéressant de découvrir un type de cellule maligne qui puisse vivre indéfiniment en culture pure sans détruire son milieu et en conservant sa malignité. En effet, pour étudier les caractères propres aux cellules néoplasiques, il fallait posséder des races pures de cellules dont la malignité ne varie pas spontanément *in vitro*. Les expériences résumées dans cette communication ont été entreprises dans le double but d'obtenir des cultures pures de fibroblastes sarcomateux du Rat et de déterminer si la race ainsi obtenue conservait indéfiniment sa malignité.

Cette étude fut faite à l'aide du sarcome 10 de la Fondation Crocker de l'Université Columbia, qui me fut donné il y a quelques mois par le Pr Francis C. Wood. Le sarcome 10 appartient au type fusocellulaire. C'est une tumeur très active qui se transmet facilement par transplantation, devient énorme, et tue les animaux sans produire de métastases. Elle se compose de cellules fusiformes, larges et courtes, entre lesquelles se trouvent ces cellules rondes, qui ressemblent à des macrophages et à des lymphocytes. Lorsqu'un fragment de cette tumeur est cultivé dans du plasma de Rat, il s'entoure d'une auréole composée d'abord de macrophages,

(1) A. Carrel. *C. R. de la Soc. de biol.*, 1925, t. XCII, p. 584.

et puis de fibroblastes. Les macrophages sont vigoureux et ne meurent pas, comme il arrive souvent dans les cultures de sarcome de Rous. Ils émigrent dans le coagulum où ils se multiplient parfois pendant des semaines. Ils ressemblent beaucoup aux macrophages normaux des tissus. Les fibroblastes, qui s'échappent ensuite de la tumeur et envahissent le milieu de culture en une nappe dense, sont identiques à cux qu'on observe dans les coupes de la tumeur. Il s'agit donc d'un sarcome fusocellulaire typique contenant, à côté des fibroblastes, un grand nombre de macrophages.

Des cultures pures de fibroblastes sarcomateux furent obtenues facilement par le procédé suivant. On plaça de petits fragments de la tumeur dans 2 c.c. de plasma de Poule dilué avec de la solution de Tyrode et contenu dans un flacon D5. Après coagulation complète, le caillot fut lavé trois fois dans 6 c.c. de solution de Ringer ou de Tyrode. Puis on introduisit dans le flacon 1 c.c. d'extrait d'embryon de Poule. Comme ce milieu est défavorable à la multiplication des macrophages du Rat et détermine au contraire celle des fibroblastes, les cellules amiboïdes diminuèrent rapidement. Au bout de quelques jours environ, le coagulum fut retiré du flacon, chaque colonie divisée en deux parties, et cultivée dans du milieu neuf. En 8 à 10 jours, les colonies doublaient en général de volume. C'est à ce moment qu'on les divisait et qu'on les plaçait dans des flacons neufs. En moins de deux semaines, les macrophages disparurent complètement et les fibroblastes demeurèrent à l'état de culture pure.

Les expériences commencérent le 19 novembre 1926, et continuent encore à l'époque où ces lignes sont écrites, c'est-à-dire le 8 avril 1927. La rapidité de croissance de ces colonies est moins grande que celle des colonies de fibroblastes normaux du Rat. Les cellules se multiplient facilement dans des milieux composés de fibrine et d'extrait d'embryon de Poule, ou des produits de l'hydrolyse de protéines de Bœuf, et ne contenant ni plasma, ni suc de tissu de Rat. Elles sont donc isolées du Rat au point de vue chimique aussi bien qu'au point de vue spatial. Depuis plus de 130 jours, ces cellules prolifèrent sans digérer le coagulum et sans mourir. De temps en temps des fragments de colonies furent inoculés à des Rats. Dans tous les cas, sans exception, une petite tumeur apparut du quatrième au sixième jour suivant l'inoculation, se développa peu à peu, et

amena finalement la mort de l'animal. Il est évident que la malignité d'une culture pure de fibroblastes du sarcome du Rat ne se modifie nullement dans les conditions que nous venons de décrire.

Il devient donc possible d'analyser les caractères des cellules malignes du Rat, aussi facilement que ceux de l'élément malin des sarcomes de la Poule, puisque le fibroblaste du sarcome Crocker n° 10 ne digère pas le milieu de culture, et reste indéfiniment normal.

[Reprinted from COMPTES RENDUS DES SÉANCES DE LA SOCIÉTÉ DE BIOLOGIE, May 6, 1927, Vol. xcvi, No. 14, pp. 1121–1124.]

AU SUJET DU SARCOME DE L'ARSENIC DE FISCHER.

PAR ALEXIS CARREL.

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L'année dernière, Fischer réussit à produire un sarcome fusocellulaire en traitant par une solution diluée de pentoxyde arsénieux une culture de rate d'embryon de Poule (1*). Cette tumeur, inoculée à des Poules, se développa localement et détermina l'apparition de métastases pulmonaires et la mort. Il est inutile d'insister sur l'importance de ce fait. On doit rapprocher ce résultat de ceux obtenus auparavant par l'injection *in vivo* de substances chimiques variées, indol (2*), oxyde arsénieux (3*) et peptone. Ces substances déterminèrent parfois, chez la Poule, des sarcomes fusocellulaires plus ou moins malins qui pouvaient se propager comme le sarcome de Rous par un virus ultramicroscopique. L'objet des expériences résumées dans cette note était de comparer les propriétés du sarcome de Fischer à celles des autres sarcomes d'origine chimique et à chercher si cette tumeur est transmissible par un agent filtrant.

Le 18 décembre 1926, Fischer prépara des cultures en gouttes pendantes du sarcome de l'arsenic dans les laboratoires à l'Institut Kaiser Wilhelm, à Berlin, les plaça à l'étuve pendant 24 heures, puis les confia au D[r] Kirby, qui voulut bien me les apporter à New-York. Je reçus ces cultures le 1[er] janvier 1927. Malgré la longueur du voyage, les cultures était demeurées vivantes. Les tissus de deux cultures, dont le coagulum était presque complètement digéré, furent immédiatement transplantés dans un milieu neuf et ne tardèrent pas à s'entourer d'une couronne de cellules. Ces cellules manifestèrent immédiatement leur propriété de digérer la fibrine du caillot. Au bout de quelques jours, on transféra les tissus dans des flacons D-5 où ils furent cultivés avec des fragments de rate. Actuellement,

(1*) A. Fischer. *C. R. de la Soc. de biol.*, 1926, t. XCIV, p. 1217.
(2*) A. Carrel. *C R. de la Soc. de biol.*, 1925, t. XCIII, p. 1278.
(3*) A. Carrel. *C. R. de la Soc. de biol.*, 1925, t. XCIII, p. 1083.

c'est-à-dire plus de trois mois après son arrivée à New-York, le sarcome se développe activement *in vitro*.

Cette tumeur possède des propriétés culturales qui permettent de la distinguer du sarcome de Rous et des autres sarcomes de l'arsenic. Son caractère principal est de digérer le coagulum avec une très grande rapidité. Au bout de peu de temps, le caillot se perce de trous faits comme à l'emporte-pièce. Ces trous ont des bords nets qui ne sont pas bordés d'une lisière blanchâtre, comme dans les cultures du sarcome de Rous. Leurs dimensions sont généralement très grandes. Parfois, la moitié du coagulum se liquéfie en un jour. Les cultures de nos sarcomes de l'arsenic montraient généralement plusieurs petites ulcérations à bord nécrosé qui leur donnaient l'aspect d'une étoffe rongée par les mites. Dans le sarcome de Fischer, le coagulum ressemble plutôt à une étoffe brûlée par des gouttes d'acide. A cause de la destruction rapide de la fibrine par les tissus, il faut, pour maintenir ces cultures en vie, ajouter souvent dans les flacons du plasma frais et des fragments de tissus normaux. Il semble donc que le sarcome de Fischer se distingue des autres sarcomes par une abondante production de ferments protéolytiques et par la tendance des cellules qui le composent à mourir en grand nombre.

Six cultures furent inoculées à des époques différentes, à huit Poules. Chez sept animaux, des sarcomes se produisirent. Dans cinq cas, les animaux moururent avec des métastases dans les poumons seuls, ou dans les poumons, le foie ou la rate. La durée moyenne de l'évolution de la maladie fut 32 jours. Des fragments de ces tumeurs furent inoculés à huit autres Poules. Dans tous les cas, une tumeur apparut et tua les animaux après une période moyenne de 20 jours. La durée la plus courte fut de 16 jours. Cette tumeur est donc moins maligne que le sarcome de Rous et que les sarcomes produits *in vivo* par l'arsenic. Au point de vue macroscopique, elle ressemble beaucoup au sarcome de Rous, mais se nécrose de façon plus prononcée et plus fréquente. Elle se compose de cellules fusiformes, dans l'intervalle desquelles se disposent de façon irrégulière des cellules rondes. Elle diffère peu, au point de vue histologique, des autres sarcomes de la Poule.

A quatre reprises différentes, des extraits aqueux de la tumeur furent filtrés dans un filtre Berkefeld et inoculés à huit Poules en

même temps qu'un peu de pulpes embryonnaire. Dans six cas, l'inoculation ne produisit pas de tumeur, ou une tumeur qui rétrocéda rapidement. Cependant, chez une Poule, l'extrait filtré détermina l'apparition d'un sarcome typique et, chez une autre Poule, d'un tératome dont une partie était sarcomateuse. Il est donc évident que le sarcome de Fischer renferme un agent filtrant, mais la propagation de la tumeur par cet agent se fait beaucoup plus difficilement que dans le cas de nos sarcomes de l'arsenic.

Il semble donc que le sarcome de Fischer se distingue du sarcome de Rous et des autres sarcomes de l'arsenic par une malignité moindre. Jusqu'à présent, il n'a pas amené la mort des Poules en moins de 16 jours. Il ne se transmet pas de façon constante par son extrait filtré. A la vérité, il n'existe aucune différence profonde entre cette tumeur et les autres tumeurs d'origine chimique de la Poule. Il est important de remarquer que ce sarcome contient un "virus" comme celui du sarcome de Rous et des sarcomes fusocellulaires que nous avons produits chez la Poule à l'aide de l'arsenic, de l'indol et du goudron. Seuls, le sarcome du goudron de Murphy et Land steiner n'a pas pu être transmis par un agent filtrant (4*). Peut-être existe-t-il une relation entre l'activité du "virus" et la malignité de la tumeur. Il est possible également que l'emploi d'une technique un peu différente aurait permis de déceler la présence d'un "virus" dans le sarcome de Murphy et Landsteiner. On peut donc considérer que le sarcomes d'origine chimique ne diffèrent pas des sarcomes spontanés de la Poule. La grande simplicité de la technique employée par Fischer dans la production de son sarcome rend d'autant plus important le résultant qu'il a obtenu.

(4*) J.-B. Murphy et K. Landsteiner. *Journ. of exper. medic.*, 1925, t. XLI, p. 807.

[Reprinted from COMPTES RENDUS DES SÉANCES DE LA SOCIÉTÉ DE BIOLOGIE, May 13, 1927, Vol. xcvi, No. 15, pp. 1198–1200.]

LA CYTOLOGIE NOUVELLE.

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L'étude des cultures pures a montré que chaque type cellulaire se caractérise, non seulement par son aspect morphologique, mais aussi par un ensemble de propriétés physiologiques spéciales. Ces propriétés étaient restées inconnues jusqu'à présent, parce qu'elles se dissimulent dans la complexité extrême des phénomènes dont, au sein de l'organisme, les tissus sont le théâtre. Quand les techniques modernes de la culture des tissus permirent d'étudier des colonies composées d'un seul type cellulaire et grandissant dans un milieu de composition connue, les potentialités cachées des cellules devinrent apparentes, et l'analyse de leurs caractères fondamentaux put être commencé. Il en résulta une modification profonde de la cytologie classique. La cytologie nouvelle ne se contente pas d'étudier la morphologie des organes nucléaires et cytoplasmiques. Elle cherche aussi les relations qui existent entre l'aspect anatomique des cellules, l'état de leur activité et les conditions physico-chimiques de leur milieu. Elle établit l'inventaire de leurs propriétés physiologiques fondamentales. Enfin, elle conduit à l'étude du mécanisme de l'association des tissus entre eux et avec les humeurs, c'est-à-dire, à la connaissance des lois de la sociologie cellulaire. Conçue de cette manière, la cytologie deviendra la base de la physiologie des Métazoaires.

Le développement de cette nouvelle science dépend entièrement de la possibilité de maintenir à l'état de culture pure les types cellulaires principaux. On sait que les tissus de la Poule se sont prêtés facilement à l'existence *in vitro* et qu'on a déjà obtenu des cultures pures de plusieurs d'entre eux: fibroblastes, monocytes du sang, épithélium de l'iris (Fischer), épithélium thyroïdien (Ebeling), épithélium du cristallin (Kirby), macrophages des tissus adultes, cellules cartilagineuses (Fischer), macrophages sarcomateux, fibroblastes sarcomateux, etc. On peut se procurer facilement des cul-

tures pures d'épithélium malpighien du Cobaye. Grâce à des techniques nouvelles, les fibroblastes normaux ou sarcomateux du Rat se cultivent aussi bien que ceux de la Poule. Comme l'ont montré autrefois Losee et Ebeling (1), il est possible d'obtenir des cultures pures de fibroblastes humains. Si le nombre de types cellulaires qui ont été étudiés jusqu'à présent à l'état pur est encore petit, il faut en attribuer la cause au nombre extrêmement restreint des expérimentateurs qui ont eu le courage d'apprendre les techniques délicates qui constituent la culture des tissus dans sa forme moderne. Ces techniques ne sont employées aujourd'hui que dans un ou deux laboratoires en Amérique et un seul en Europe. Il est probable que la plupart des cellules peuvent être cultivées à l'état pur. Mais il faut qu'un nombre suffisant de travailleurs abordent ce problème.

Toutes les données de la cytologie classique peuvent être utilisées dans l'édification de la cytologie nouvelle, à condition qu'une relation soit établie entre l'aspect morphologique des cellules et leur état fonctionnel. Quand on étudie des cellules extirpées à un animal vivant, ou provenant de la culture impure de tissus par les anciennes techniques, comme le font Lewis, Burrows, Loeb, Champy, Maximoff, Drew, Borrel, etc., il est impossible de connaître l'état physiologique de ces cellules et, par suite, la signification de ce qu'on observe. Ce n'est qu'à l'aide de colonies composées d'un seul type cellulaire, placées dans des flacons contenant un milieu de composition connue, et manifestant une forme mesurable d'activité qu'on peut établir une relation entre les états morphologique et fonctionnel du noyau des organes cytoplasmiques. Cette méthode permet de distinguer les changements qui correspondent à des modifications métaboliques de ceux qui sont dus à des causes plus profondes. La transformation des monocytes du sang en macrophages des tissus, par exemple, ou le retour des macrophages à un état voisin de celui des monocytes se produisent facilement sous l'influence de variations dans la nature et la concentration des substances nutritives du milieu. Mais la métamorphose des macrophages en fibroblastes, que nous avons observée souvent, et celle des fibroblastes en macrophages réalisée récemment par Fischer, sont causées par des substances d'une autre nature.

(1) J. R. Losee et A.-H. Ebeling. *Journ. of exper. Medic.*, 1914, t. XIX, p. 593.

La tâche principale de la cytologie nouvelle est de découvrir les propriétés physiologiques qui caractérisent chaque type cellulaire. Il est impossible d'aborder l'étude de ces propriétés par une autre méthode que celle des cultures pures. Elle seule permet de nodifier avec précision les conditions de la vie des colonies, et de mettre en lumière les potentialités des cellules qui restent le plus souvent cachées pendant leur vie normale. En bactériologie, on ne se contente pas d'étudier la forme et la réaction des microbes à l'égard de certaines matières colorantes, mais on examine aussi l'aspect de leurs colonies, leur effet sur le milieu de culture, les poisons qu'elle sécrètent, leur susceptibilité aux différents antiseptiques, les substances nutritives qu'elles réclament, etc. Il en est de même des cellules des tissus. Nous pouvons aujourd'hui identifier ces cellules, non seulement par leur structure et les réactions tinctoriales de leurs organes, mais par l'apparence de leurs colonies, leur mode de relations, l'allure de leur locomotion sur les films cinématographiques, leur effet sur le coagulum du milieu, leur taux de croissance, la nature et la concentration des substances chimiques qui sont toxiques pour elles, la nature des substances qui produisent leur multiplication, etc. Ainsi, il devient possible d'acquérir des tissus une connaissance beaucoup plus étendue et plus fondamentale que celle qui nous a été fournie jusqu'à présent par la morphologie. La cytologie nouvelle permet non seulement d'identifier les cellules, mais de prédire leur conduite dans des conditions déterminées. Elle dévoile les propriétés spécifiques de chaque type cellulaire. Grâce à elle, le mécanisme des phénomènes complexes qui se passent dans les tissus normaux ou pathologiques peut être soumis à l'analyse expérimentale. Sa fécondité sera nécessairement beaucoup plus grande que celle de la cytologie classique.

[Reprinted from COMPTES RENDUS DE SÉANCES DE LA SOCIÉTÉ DE BIOLOGIE, May 13, 1927, Vol. xcvi, No. 15, pp. 1201–1203.]

LES PROPRIÉTÉS SPÉCIFIQUES DES FIBROBLASTES D'UN SARCOME DU RAT.

PAR ALEXIS CARREL.

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Le but des expériences, résumées dans cette note, était d'étudier, suivant la conception nouvelle de la cytologie (1*), les propriétés qui distinguent les fibroblastes sarcomateux à l'état de culture pure des fibroblastes normaux. C'est, en effet, par la connaissance des caractères physiologiques apparents et des potentialités cachées des principaux types de cellules malignes que le mécanisme de la croissance des tumeurs sera dévoilé. Déjà, l'étude du macrophage des sarcomes de la Poule a montré que cette cellule diffère du type normal par la faculté de produire des ferments protéolytiques en abondance et de mourir au lieu de se diviser (2*). Mais la malignité n'est peut-être pas toujours due à une même cause. Aussi est-il nécessaire d'examiner les caractères de cellules néoplasiques de types variés. Dans cette note, je me bornerai à décrire les propriétés des fibroblastes malins du sarcome 10 de la Fondation Crocker de l'Université Columbia (3*) que je possède depuis plus de quatre mois à l'état de culture pure, et à comparer ces cellules aux fibroblastes normaux.

Les relations des fibroblastes malins les uns avec les autres et l'aspect de leurs colonies ont été étudiés dans des flacons D-5 ordinaires et dans une boîte spéciale où ils ont été cinématographiés. Les cellules s'accolent étroitement les unes aux autres, entrent en contact par leurs faces latérales aussi bien que par leurs extrémités, et forment un véritable tissu. Elles se distinguent ainsi nettement des macrophages sarcomateux, et ne diffèrent pas des fibroblastes normaux. Elles sont généralement allongées en forme de palmes formant

(1*) A. Carrel. *C. R. de la Soc. de biol.*, 1927, t. XCVI, p. 1198.

(2*) A. Carrel. *Journ. of the Amer. Med. Assoc.*, 1925, t. LXXXIV, p. 157; *C. R. de la Soc. de biol.*, 1925, t. XCII, p. 584.

(3*) A. Carrel. *C. R. de la Soc. de biol.*, 1927, t. XCVI, p. 1119.

comme une haie épaisse. Elles peuvent aussi être larges et courtes, et se disposer en mosaïque, comme sur les coupes de la tumeur elle-même. Elles paraissent plus grossières et plus dures que les cellules normales. On les voit traverser facilement des fragments de coagulum qui arrêtent habituellement la migration des fibroblastes normaux. L'examen des films cinématographiques montre qu'il n'y a pas de différence fondamentale entre le mode de locomotion et l'agencement des fibroblastes sarcomateux et normaux. Les colonies ne renferment pas de cellules mortes. Elles sont composées uniquement d'éléments actifs. C'est un caractere qui les différencie profondément des colonies de fibroblastes sarcomateux de la Poule.

Les fibroblastes sarcomateux cultivés dans un coagulum lavé de plasma de Poule ne digèrent jamais la fibrine. Le milieu reste indéfiniment intact. Au contraire, les fibroblastes sarcomateux de la Poule détruisent plus ou moins rapidement le coagulum. Quand, au lieu de cultiver les fibroblastes de Rat dans un coagulum de plasma de Poule, on les place dans du plasma de Rat, la digestion de la fibrine se produit. Les fibroblastes sarcomateux exercent donc sur la fibrine du plasma de Rat une action plus marquée que celle des fibroblastes normaux.

La rapidité de croissance des colonies se mesure en dessinant à l'aide d'un projectoscope l'accroissement de leur surface, dans un milieu déterminé. Ce milieu se compose de 2 c.c. de coagulum lavé, à la surface duquel on place 1 c.c. d'un extrait d'embryon de Poule dont la teneur en azote varie de 0.045 gr. à 0.055 gr. par 100 c.c. Les tissus se trouvent donc dans un liquide qui contient approximativement de 15 à 18 mgr. d'azote par 100 c.c. La surface des colonies double en cinq jours environ. Leur accroissement est moins rapide que celui des fibroblastes de Poule et de Rat cultivés dans le même milieu.

Les fibroblastes sarcomateux ne se multiplient pas dans le plasma dilué du Rat. Ils prolifèrent activement dans l'extrait d'embryon de Poule, et dans les premiers produits de l'hydrolyse de la glande pituitaire de veau, et du testicule de Taureau. L'énergie résiduelle des colonies, mesurée par l'étendue et la durée de la migration des cellules dans la solution de Tyrode, est faible. En moins d'une semaine, les fibroblastes épuisent généralement leurs réserves. Il

n'existe donc aucune différence entre les cellules normales et sarcomateuses au point de vue de l'utilisation des substances contenues dans les milieux habituels. La nature du milieu de culture et la rapidité de la croissance des colonies ne paraissent pas modifier leur malignité. Des tissus cultivés soit dans du plasma ou de la solution de Tyrode, ou dans du suc d'embryon, et se trouvant par suite dans des états très différents d'activité, produisent toujours des tumeurs lorsqu'on les transplante à des Rats. Mais en général, les tumeurs apparaissent plus rapidement quand les colonies ont été privées de nourriture dans la solution de Tyrode que si leur croissance a été arrêtée par un séjour dans du plasma. Dans aucun cas, il ne fut possible de "guérir" les fibroblastes de leur malignité par des changements de leur état métabolique. Jusqu'à présent, les colonies sont restées malignes dans tous les milieux où elles ont été cultivées.

Le fibroblaste malin du sarcome 10 de la Fondation Crocker possède l'apparence d'une cellule normale. Il n'est jamais malade. Au lieu de mourir, il se divise indéfiniment sous l'influence des mêmes substances chimiques qui déterminent la division du fibroblaste normal. Mais pas plus que lui, il ne peut se nourrir de plasma dilué. Il est donc impossible de comprendre pourquoi il se multiplie indéfiniment au sein de l'organisme, tandis que le fibroblaste normal ne le fait pas. On doit supposer qu'il rencontre, après sa transplantation dans les tissus du Rat, certaines conditions qui n'existent pas *in vitro*. Une analyse plus approfondie de ce phénomène nous donnera peut-être l'explication de son mécanisme.

[Reprinted from COMPTES RENDUS DES SÉANCES DE LA SOCIÉTÉ DE BIOLOGIE, June 17, 1927, Vol. xcvii, No. 19, pp. 19–21.]

LE RÔLE DES MACROPHAGES DANS LA CROISSANCE D'UN SARCOME DU RAT.

PAR ALEXIS CARREL.

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Le sarcome 10 de la Fondation Crocker de l'Université Columbia est une tumeur composée de cellules fusiformes, larges et courtes, entre lesquelles s'interposent un assez grand nombre de cellules rondes qui, sur les coupes, ressemblent à des macrophages. Lorsqu'on cultive des fragments de cette tumeur dans un coagulum de fibrine et un milieu liquide composé de plasma hépariné de Rat, une grande quantité de macrophages s'échappent d'abord du tissu. Puis, des fibroblastes apparaissent. Ils sont semblables à ceux que l'on aperçoit dans les sections de la tumeur. Quand le milieu est composé de suc embryonnaire, ou de protéoses de la glande pituitaire, la migration des macrophages est beaucoup moins abondante, et les fibroblastes envahissent rapidement le caillot où ils forment un tissu dense. Si, après avoir enlevé le tissu originel qui occupe le centre de la colonie, on se sert exclusivement de suc embryonnaire de Poule comme milieu liquide, on obtient en peu de temps des cultures de fibroblastes, dont les macrophages sont complètement absents. Ces colonies de fibroblastes à l'état pur ne perdent pas leur malignité. Si on les inocule à des Poules, elles déterminent toujours des tumeurs qui deviennent énormes et amènent la mort de l'animal par cachexie, sans produire de métastases.

Ces fibroblastes malins demandent pour leur multiplication les mêmes substances que les fibroblastes normaux. Ils prolifèrent abondamment dans le suc embryonnaire et dans les protéoses de la glande pituitaire et du foie, et ne se multiplient pas dans le plasma dilué du Rat. Il est difficile d'expliquer leur croissance indéfinie *in vivo* au sein de la lymphe interstitielle qui ne permet pas la multiplication des fibroblastes normaux. Il faut donc admettre l'existence d'un facteur accessoire qui leur permet de se procurer, dans les humeurs d'un animal adulte, l'azote dont ils ont besoin pour la

synthèse de nouvelles cellules. Ce facteur peut être une substance spéciale présente dans la lymphe interstitielle, ou sécrétée par les macrophages qui sont un élément constitutif du sarcome 10, ainsi que d'autres sarcomes fusocellulaires. Les macrophages pourraient agir par leur sécrétions propres, ou par la production, à l'aide de leurs ferments, de protéoses et de peptones aux dépens des protéines. Afin de déterminer si la lymphe interstitielle contient des substances activantes, j'ai placé pendant 24 heures une culture pure de fibroblastes malins dans le tissu cellulaire sous-cutané du Rat. Au bout de ce temps, la culture fut replantée dans un flacon contenant le milieu habituel. On constata que les fibroblastes avaient perdu une grande partie de leur énergie de croissance. Ce ne fut qu'après avoir été cultivées plusieurs jours dans du suc embryonnaire que les colonies reprirent leur activité normale. En somme, les fibroblastes, pendant leur séjour dans le tissu sous-cutané du Rat, n'avaient reçu de la lymphe aucune substance nutritive. Ils avaient même perdu la plus grande partie de leur activité. Mais, si les cultures étaient laissées plus longtemps dans l'animal, quatre jours par exemple, une petite tumeur se développait. Dans cette tumeur, les fibroblastes n'étaient plus seuls. Ils vivaient en symbiose avec des macrophages. Ils avaient, semble-t-il, attiré à eux les cellules migratrices de l'hôte qui, dans ce cas, ne représentaient nullement une réaction de défense de l'organisme. Il semble donc que le facteur, permettant aux fibroblastes de se multiplier au sein d'humeurs non nutritives et inhibitrices, soit le macrophage. Nous savons déjà que des colonies de fibroblastes de Poule, cultivées dans du plasma, s'accroissent quand on place auprès d'elle un petit fragment de rate. Il est possible qu'un mécanisme semblable puisse expliquer la croissance du sarcome du Rat. C'est pourquoi j'ai cherché si des fibroblastes malins, cultivés dans un milieu non nutritif, se multiplient quand on leur adjoint des macrophages normaux.

Dans des flacons D-5, contenant du plasma de Poule dilué avec de la solution de Tyrode et un peu de suc embryonnaire, on plaçait à une grande distance l'une de l'autre les deux moitiés d'une colonie de fibroblastes sarcomateux à l'état pur. A l'un des fragments, on joignait un petit fragment de moelle osseuse de Rat. Après coagulation du plasma, le caillot était lavé avec de la solution de Tyrode

pendant 30 minutes. Puis, on injectait dans le flacon le milieu liquide qui se composait de plasma hépariné de Rat. La croissance des colonies était mesurée par le méthode habituelle. Au bout de quelques jours, la colonie isolée s'était à peine accrue. Autour d'elle, des cellules avaient émigré dans le milieu de culture. Mais la prolifération était faible et s'arrêtait bientôt. Au contraire, la colonie qui était partiellement entourée par les macrophages de la moelle devint beaucoup plus active. Ses dimensions augmentèrent de façon considérable. Il était indiscutable que la présence de macrophages déterminait la multiplication des fibroblastes sarcomateux cultivés dans du plasma, bien que le plasma soit pour les fibroblastes un milieu à la fois non nutritif et inhibiteur.

On peut donc croire que les macrophages qui vivent en symbiose avec les cellules fusiformes dans le sarcome du Rat agissent sur les fibroblastes de la même façon qu'*in vitro*. Les macrophages représenteraient le facteur complémentaire qui permet à l'élément malin de se multiplier dans les humeurs de l'animal adulte et de produire une tumeur. Ils atteindraient ce résultat soit en sécrétant des protéines analogues à celles du suc embryonnaire, soit en déterminant par leurs ferments protéolytiques la digestion incomplète des protéines de la lymphe ou des tissus. L'avenir déterminera quelle part de vérité contiennent ces hypothèses.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1927, Vol. xlvi, No. 1, pp. 1–6.]

SURFACE TENSION OF SERUM.

XV. THE THICKNESS OF THE MONOLAYER OF RABBIT PLASMA.

BY P. LECOMTE DU NOÜY, SC.D.

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(Received for publication, April 5, 1927)

In the preceding papers of this series,[1] it has been shown that a well defined minimum value of the static surface tension of fresh, clear serum could be observed at a given high dilution, and the conclusion was reached that under given conditions serum and serum solutions behave as true solutions. In order to explain the presence of this minimum,—or of these minima, as in most cases more than one can be observed,—it was necessary to resort to the hypothesis that all the constituents of the serum acted as though they were bound together in the shape of a huge molecule and that at some critical concentrations, the organization of these molecules in the surface layer decreased the value of the tension measured by the du Noüy tensiometer. The similarity of this phenomenon with that observed in the case of sodium oleate,[2] egg albumin,[3] and recently digitonin,[4] the important decrease in the rate of evaporation at the same critical concentration,[5] and the displacement of the minimum when the surface of adsorption was changed,[6] seemed to bear out this hypothesis satisfactorily.

The question then arose as to whether solutions of plasma would behave in the same way, namely, as large molecules capable of organizing themselves at a given concentration, or whether they would behave as though molecules of another substance but of approximately the

[1] du Noüy, P. L., Surface tension of serum, Papers I–X, *J. Exp. Med.*, 1922, xxxv, to 1924, xl; Paper XI, *J. Gen Physiol*, 1924, vi, 625; Papers XII–XIV, *J. Exp. Med.*, 1925, xli to xlii.

[2] du Noüy, P. L., *Phil. Mag.*, 1924, xlviii, 264, 664.

[3] du Noüy, P. L., *J. Biol. Chem.*, 1925, lxiv, 595.

[4] Unpublished experiments.

[5] du Noüy, P. L., *J. Exp. Med.*, 1924, xxxix, 717.

[6] du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 133.

same molecular weight were added to the serum. In other words, when fibrinogen is present in the serum, does it exist separately, or is it part of the serum molecule, which would then be a "plasma molecule"?

If the fibrinogen is supposed to exist as a separate substance in solution (plasma = serum molecules + fibrinogen molecules), then the concentration at which the principal minimum of surface tension occurs will not be changed as the space occupied in the horizontal plane by the "serum molecules" will be the same. By diluting serum or plasma 10,500 times, a minimum should be expected in both cases, although the concentration of the latter in proteins would be greater, and consequently also the thickness of the adsorbed layer. The same would happen if the fibrinogen were bound at one end of the serum molecule, without involving any change in structure. Therefore, in case no shift should be observed, no definite answer could be given as to whether the fibrinogen exists separately or whether it is merely an easily detachable group fixed at one end of the serum molecule.

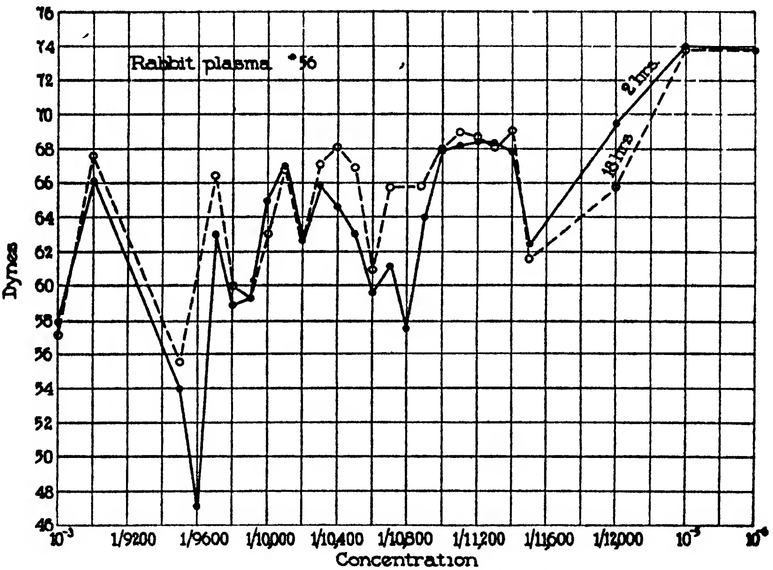
But if the fibrinogen, instead of being merely additive, were actually part of the molecule, the whole structure and symmetry of the assumed "plasma molecule" might be different from that of the "serum molecule," and consequently, the space occupied in the horizontal plane by these two molecules might also differ. If it is assumed that the "plasma molecule" can orient itself in a monolayer as does the "serum molecule," a shift in the place of the minimum is to be expected. Therefore, should a minimum be observed with plasma solutions, and should this minimum occur at the same concentration as it does with serum, it would indicate that the orientation and dimensions of the serum molecules in the horizontal plane are unchanged. In this case, no answer could be given as to the possible link between fibrinogen and serum; on the contrary, should the principal minimum be shifted toward higher or lower concentrations, one could logically assume that the molecules present in the solution are different in size and shape from those of the serum, but similarly capable of organizing as polarized units.

EXPERIMENTAL AND RESULTS.

The blood was collected and centrifuged immediately, and the plasma was diluted as soon as possible in saline solution. In a few

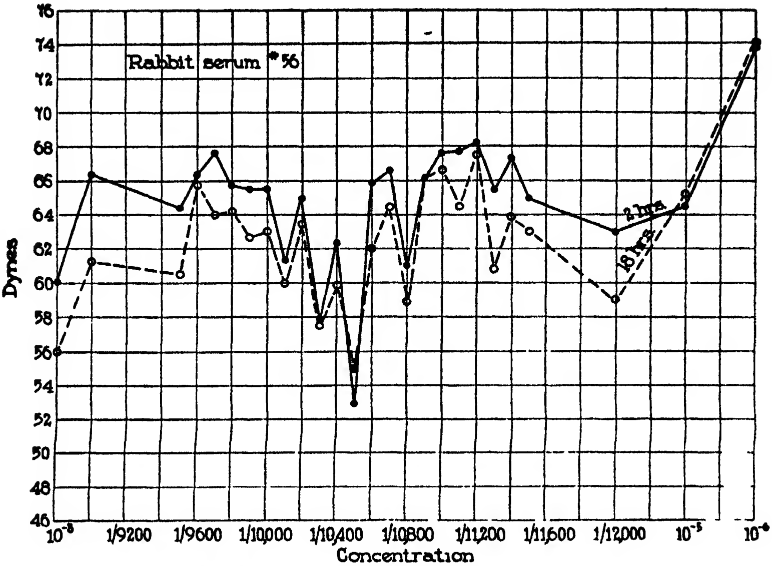
cases, coagulation occurred at the concentration 1:100, but never at 1:1000. When dilutions were made rapidly, no difficulty was encountered. The experimental part of this work was carried out by Mr. J. Zwick. (Text-figs. 1 and 2.)

The results of twenty-two series of measurements are not as sharply defined as one might have hoped. However, when the frequency of occurrence of the minima at a given concentration is taken into consideration (Text-fig. 3), it is clear that minima are observed in the case of

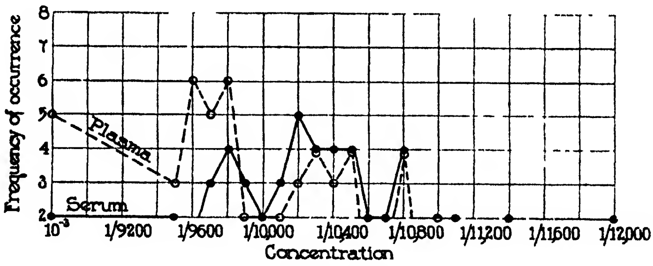


TEXT-FIG. 1. Experiment 1. Static values of diluted rabbit plasma, as a function of the concentration The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.

plasma and that the most important of them are shifted with respect to those of the serum. Text-fig. 4 expresses the mean value of the twenty-two curves. If the minima which only occurred twice or less are omitted as due to experimental errors, it is obvious from Text-fig. 3 that out of thirty-eight minima observed in the eleven series of experiments with plasma, twenty or 53 per cent occurred at concentrations between 1:9500 and 1:9800. Out of the thirty-four minima found for



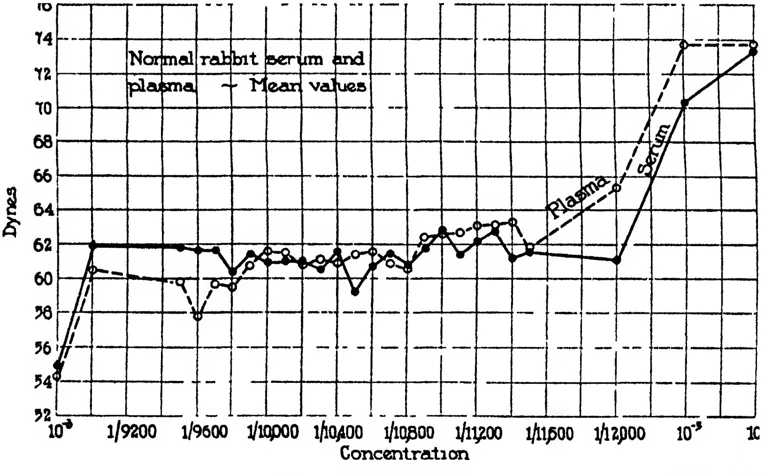
TEXT-FIG. 2. Experiment 2. Static values of diluted rabbit serum, as a function of the concentration. The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.



TEXT-FIG. 3. Frequency of occurrence of the minima of static surface tension in plasma and serum (eleven experiments for each).

serum under the same conditions, seventeen or 50 per cent occurred at concentrations between 1:10,200 and 1:10,500. Although the minima 1:9600 for plasma and 1:10,500 for serum are quantitatively more important, as is seen from Text-figs. 3 and 4, one must not neglect the fact that the minimum 1:9800 (plasma) shows the same

degree of probability (Text-fig. 3), and that the minimum 1:10,200 (serum) was even more frequent than that at 1:10,500. Therefore, it seems desirable to take into account the mean values, namely, 1:9700 for plasma, and 1:10,350 for serum. These concentrations differ by about 6.3 per cent. This percentage expresses the difference in thickness of monolayers of plasma and of serum. It is assumed that the specific gravity of fibrinogen is the same as that of the other proteins of the serum, which is probably very nearly true. Hence, the thickness of the plasma monolayer is greater by 6.3 per cent in



TEXT-FIG. 4. Mean values of eleven series of measurements for serum and eleven series of measurements for plasma.

round figures than that of serum. As the "length" of the total "serum molecule," according to our former determinations,[6] is about 40.5 Ångström units, or 4.05mμ the length of the "plasma molecule" will be 43.05 Ångstroms in round figures, or 4.3mμ. This difference should correspond to the amount of fibrinogen present. Two careful determinations of the fibrin content of rabbit plasma, with two different methods,[7] gave the figures 4.5 and 4.65 per cent of total proteins.

[7] Van Slyke's method, and the method consisting of weighing the dry substances of the plasma and of the serum, after dialyzing the salts out, then the fibrin, proper care being taken to remove all the salts by washing the fibrin carefully.

According to Lambling,[8] only 60 to 70 per cent of the fibrinogen is transformed into fibrin after coagulation. If 65 per cent is taken as a mean value, 4.6 per cent of fibrin corresponds to about 7 per cent of fibrinogen. The shift in the minimum, therefore, seems to be in good accord with the fibrinogen content, rather than with the fibrin content. However, it is not the intention of the writer to insist upon his figure as being a check of Lambling's statement, as too little is known at present about this subject. The error introduced through the fact that the amount of fibrinogen is expressed in per cent of the proteins and that the increase in thickness of the monolayer is expressed in per cent of total serum does not amount to more than about 0.3 per cent and is negligible in consideration of the other causes of error.

CONCLUSIONS AND SUMMARY.

Experiments are reported which indicate that a shift toward higher concentrations is observed in the minimum value of the static surface tension when plasma instead of serum solutions is used. The amount of the shift, expressed as a function of the concentration, shows that the figures are in satisfactory agreement with the determined amount of fibrinogen in the plasma.

Some evidence is given that "plasma molecules" capable of organizing themselves on adsorbing surfaces exist in plasma, and that their length would be approximately 4.3 mμ in round figures, instead of 4.0 mμ for the serum. The area occupied in the plane of adsorption by one individual molecule is, however, smaller than that occupied by the "serum molecule," thus indicating a marked structural difference between the two, the "plasma molecule" being narrower but longer than the "serum molecule." This difference may be due either to a different orientation accompanied by an increase in one of the dimensions, or else to an actual difference in structure with respect to the main axis, resulting in a decrease in the mean diameter of the "serum molecule" with an increase in the length of its main axis. The mass of the "plasma molecule" is about 6.3 per cent larger than that of the "serum molecule," in the case of rabbit serum.

[8] Lambling, E., Précis de biochimie, Paris, 1911, 250

[Reprinted from The Journal of the American Chemical Society, July, 1927, Vol. xlix, No. 7, pp. 1710–1717.]

THE MOVING-BOUNDARY METHOD FOR DETERMINING TRANSFERENCE NUMBERS.

VI. Further Developments in Experimental Technique.

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(Received for publication, May 11, 1927.)

1. INTRODUCTION

Many of our experimental methods in the use of the moving boundary for measuring transference numbers have been described in the earlier papers of this series [1] Recently, however, we have made a number of changes and improvements in the apparatus that seem worthy of record as they have resulted in greatly increased accuracy and convenience, in the possibility of use of the method through wider ranges of concentration and materials, and in the use of the method under unfavorable circumstances.

2. CHANGES IN THE DESIGN OF THE CELL

All of our recent work has been carried out with the type of cell designed by MacInnes and Brighton[1c] in which the boundary is formed by means of a shearing motion. The improved apparatus is shown in Fig. 1. The disks B and C are now made of plate glass, instead of the hard rubber formerly used. The glass does not warp after a little use as the rubber did. It also has a smoother surface, and its transparency is frequently of service. The channels, etc., in the surface of the plates that are in contact were etched out with hydrofluoric acid.

[1] (a) MacInnes and Smith, This Journal, **45,** 2246 (1923). (b) Smith and MacInnes, *ibid*, **46,** 1398 (1924) (c) MacInnes and Brighton, *ibid.*, **47,** 994 (1925). (d) Smith and MacInnes, *ibid*, **47,** 1009 (1925). (e) MacInnes, Cowperthwaite and Blanchard, *ibid.*, **48,** 1909 (1926).

Since the motion of a boundary through 10 cm. of the measuring tube involves a volume of only 1.7 cc., and in our most recently constructed cell only 0 32 cc , it is evident that all precautions must be taken to prevent slight volume changes in the apparatus that would, ever so slightly, affect the motion of the boundaries. In pressing the lubricated plates together a small amount of air was included under a slight excess of pressure. This was occasionally sufficient to form a bubble at the junction of the measuring tube and apparently produced irregularities in the readings. This difficulty was overcome by placing a vent, F in Fig. 1, which releases the pressure by leading off the excess of air from the space between the plates to a point above the level of the thermostat water

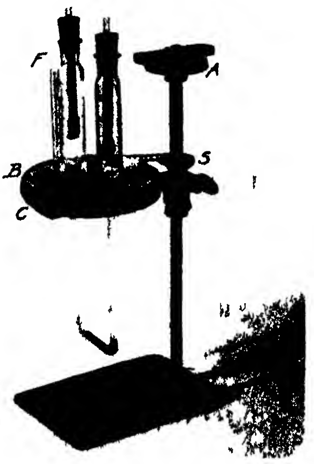

Fig 1

It has been our practice to have one of the electrode vessels completely filled with solution. The moving-boundary results are corrected, according to the method of Lewis,[2] for volume changes at this closed end of the apparatus The other electrode vessel contains a bubble of air to allow for possible expansions and contractions during the electrolysis. Originally this bubble was at the top of the vessel which projected into the air and was thus exposed to changes of temperature This was probably the cause of some irregularities in our measurements At present, however, one of the tubes holding the electrodes is left hollow and holds a small volume of air under the solution where it encounters a uniform temperature.

[2] Lewis, This Journal, 32, 862 (1910).

An added convenience, which makes possible the turning Disk B over Disk D with a minimum of mixing due to shaking, is furnished by the chain and sprocket arrangement also shown in Fig. 1. The brass Disk A which extends beyond the water of the thermostat is connected to a small sprocket S by means of a brass tube which encircles the upright of the stand. The sprocket engages a chain which is passed around Disk B. The chain is held in place by means of a rubber band which is slipped over a strip of brass holding a pin which, in turn, is inserted into a hole bored in the glass disk. Thus by turning the brass Disk A, the glass Disk B can be swung into place.

The convenience in measuring the time required in the passage of a boundary between graduations of the measuring tube has been greatly increased by substituting a Veeder magnetic counter for the watch previously used. The counter is operated once per second, by an accurate pendulum clock. Electrical contacts with the pendulum have been avoided by making the pendulum interpose a screen between a light and photo-electric cell once per second. By amplification this is made to operate the electric counter. The latter is started when the boundary passes the first graduation mark, and readings are made directly in seconds thereafter. A large amount of burdensome computation (subtractions, and changing of hours and minutes into seconds) is thus avoided.

3. THE EFFECT OF VIBRATION

In our earlier experiments in Cambridge we found that erratic results were obtained if there were a large difference between the densities of the leading and indicator solutions, such as exists between silver or cesium nitrates and lithium nitrate. The boundary usually started at nearly the correct rate but gradually slowed down toward the end of the determination. This effect is undoubtedly due to mixing caused by waves, over the surface of the boundary, generated by external vibrations. By exercising ordinary care to remove sources of vibration, results were obtained even with silver solutions. However, after moving the apparatus to New York City the vibrations were found to be intense enough to make the moving-boundary method quite useless, even for pairs of solutions without large differences in density.

This serious difficulty was overcome, after a series of preliminary experiments, by the use of a modified Julius suspension for the thermostat. A diagram of the arrangement is shown in Fig. 2. The thermostat is placed on a cast-iron plate, *a*, from which four heavy bolts extend to about the level of the center of gravity of the suspended system. From these bolts extend four 4 cm. heavy coil springs, *c*, about 45 cm. long which, in turn, are supported by four light ropes, *d*, tied to hooks fixed in a heavy beam in the ceiling. Under these conditions no external vibrations of any strength reach the thermostat, as is shown by the steadiness of a beam of light reflected from the surface of a dish of mercury. Although these are ideal conditions for the progress of the measurements it is necessary to commence the determination, that is, to turn the electrode vessels into place over the measuring tube, without starting vibrations in the system. This we have accomplished as follows. The lower edge of the suspended system is hung very near to the table top *t*. From the center of the cast-iron plate, *a*, extends an iron rod, *f*, which passes through a hole in the table top and to which is attached a rope which is connected to a foot treadle (not shown in the figure). By pressing on this treadle the suspended system is temporarily placed in contact with the table. Choosing a moment when vibration is feeble, the boundary is made by turning Disk B (Fig. 1) on Disk C. The treadle is then carefully released. This can be done without causing the suspended system to swing.

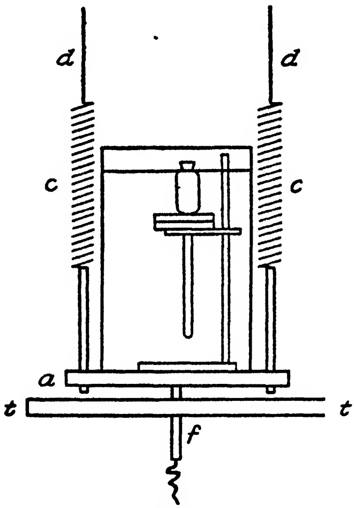

Fig. 2.

It has been our frequent observation that a boundary which starts at an incorrect rate (due presumably to a very slight initial mixing of the solutions) will gradually approach the correct rate as the experiment proceeds, unless there is an additional source of dis-

turbance. It appears, therefore, that the vibration during the time that the boundary is being formed and the suspended system is released cannot have an appreciable effect.

4. EXPERIMENTS WITH RISING BOUNDARIES

So far all our work has been carried out with indicator solutions which are lighter than the solution of which the transference number is to be measured. The indicator solution must therefore be on top of the leading solution and the boundary must descend during the determination. It seemed probable that a greater range of indicators would be available, including, possibly, some colored materials which might be visible at great dilutions, if a cell could be constructed which would utilize indicator solutions which are heavier than the measured solution. This would, of course, involve rising boundaries. Experiments were therefore carried out with an apparatus which was substantially the cell shown in Fig. 1 inverted, although a number of details had to be changed.

Observations were made on the boundaries $KMnO_4$ 0.07 $N \rightarrow$ KCl 0.1 N, and $BaCl_2$ 0.07 $N \rightarrow$ KCl 0.1 N. In both cases the boundaries were relatively sharp at the start, although somewhat convex upward. As they progressed, however, they became increasingly diffuse and instead of maintaining a steady motion as would be expected, the current being kept constant, both boundaries slowed down. It was evident that some additional condition governed the phenomenon when the boundaries were rising.

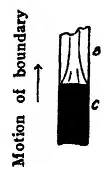

Fig. 3.

With the permanganate-chloride boundary there was another effect which was visible, due to the color of the indicator solution. A representation of the effect is shown in Fig. 3, in which the heavily shaded region, C, represents the portion of the tube containing the potassium permanganate indicator solution and the unshaded, B, the potassium chloride solution. Rising from the boundary was a faintly pink region of chimney shape, the color of which became more intense as the electrolysis proceeded, and finally not only this region but all of the potassium chloride region became colored. It immediately became of interest to find out whether a similar effect

is to be observed with a descending boundary, and it was a relief to find that with permanganate as the leading solution and an acetate as indicator, a perfectly sharp boundary was formed with no pink color in the acetate solution. Furthermore, the boundary moved at the normal uniform rate.

The explanation of the disturbing effect with rising boundaries appears to be as follows. Since the indicator solution contains ions which have lower mobility than the ions of corresponding charge in the leading solution, and is further diluted according to the relation[1a, 3] $C/T = C'/T'$, the conductance of the indicator solution must be lower than that of the measured solution. The result is therefore that due to the higher potential gradient, more heat is generated in the indicator solution than in the solution it follows. The effect shown in Fig. 3 is due to the fact that a small amount of the potassium chloride solution immediately in contact with the boundary became warmed and rose, carrying a small amount of permanganate with it. A similar effect must have occurred in the barium chloride-potassium chloride boundary, but it was not visible. In the case of the descending boundary, on the other hand, the warmer indicator solution is on top, and the difference of temperature tends to keep the two solutions separate, if it has any effect at all.

It is evident, therefore, that in all moving-boundary measurements the solution of lower conductance must be on top. Some of the early work with the moving boundary must be in error on account of failure to observe this effect. An additional effect of heat generated in the apparatus will be given in Section 6.

5. THE VISIBILITY OF THE BOUNDARY

In all our measurements we read, to the nearest second, the time when the boundary passes a given graduation mark. An increase of accuracy has been attained by graduating the tube as is shown in Fig. 4. The graduation marks extend on either side, one-fourth or less of the distance around the tube, leaving a clear space in front and back. In this way the boundary is always visible, and is never covered by the graduation mark. With faint boundaries, such as

[3] C, C', T and T' are, respectively, the concentrations and transference numbers of the measured and indicator solutions.

are obtained with dilute solutions, this arrangement is entirely necessary.

The possibility of distinguishing the junction of two transparent solutions depends upon the differences in their refractive indices. The arrangement now used for viewing the boundary is shown diagrammatically in Fig. 5. An electric lamp E is shaded by a metal screen except for a slit, a–b (seen edgewise in the figure) which is covered by a translucent screen. By means of a rack, sprockets and chain this slit can be moved vertically. Ignoring the effects of the glass walls of the thermostat M–M′ and N–N′ and of the measuring tubes, the path of a beam of light from the lower edge b of the illuminated slit is b, B, J. Thus, the edge b, viewed *through the boundary*, appears as if it were at b′. At a certain limiting value of the angle b B b′ we have the condition for total reflection. At this point the

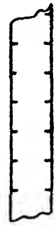

Fig 4.

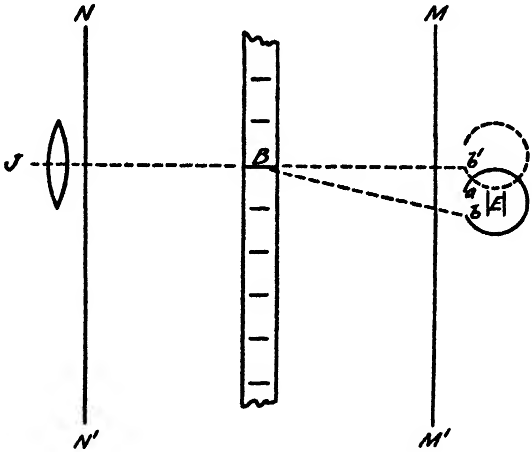

Fig. 5.

boundary appears as a sharp dark line at b′ with an illuminated area below it due to the fact that the illuminated slit can also be viewed directly through the lower solution. The apparent separation of the direct and refracted images depends on the differences of the indices of refraction of the solutions meeting at the boundary.

A very surprising increase in the visibility of boundaries is obtained by the simple device of placing a lens at the point J as shown in the figure. The lens must have a focal length reaching only to the region of the boundary. The effect of this arrangement is to throw the directly viewed edge of the slit b out of focus. The edge of total reflection, which constitutes the boundary, can thus be much more readily distinguished. Other optical systems that we have tried have been less effective than the simple one just described.

6. CONDITIONS GOVERNING "ADJUSTMENT"

As explained in previous papers in this series, the ions of the leading solution and the indicator move at the same rate when the solutions are adjusted to the condition

$$C/T = C'/T' \tag{1}$$

which must be, in general, obtained by a series of experiments. A curve of the apparent transference numbers as ordinates against the indicator concentrations C' as abscissas takes an upward slope in the dilute region, then a horizontal portion followed by a slope for more concentrated indicators. The correct transference number, and the values fulfilling the condition of Equation 1 are on the flat portion of the curve.[1d] Now the relative width of this flat portion is an important matter, since it determines the number of determinations that must be made in order to establish the shape of the curve. According to Kohlrausch[4] and others the condition represented by Equation 1 should establish itself automatically, no limits of initial concentration being stated. However, we have found in general that this can only be relied on for deviations of 3 to 5% from the correct concentration. There are apparently additional influences, including diffusion, that limit the range of adjustment. We have found, for one thing, that adjustment is considerably modified by the amount of heat developed in the measuring tube. Some experiments already published[1b] have shown that with an apparatus involving the use of a wide tube (10 mm.) the flat spot in the curve nearly disappeared. In that case, due to the relatively low ratio of

[4] Kohlrausch, *Ann. Physik*, 62, 209 (1897).

external surface to area of tube, the heat had little opportunity to be conducted away. We have recently observed much the same effect with a 6 mm. tube with more concentrated solutions, and thus necessarily higher currents than were used in the earlier experiments. On the other hand, we have found adjustment over wide ranges of indicator concentrations under two conditions, (a) very dilute solutions, and (b) solutions of electrolytes having high equivalent conductances. In the first of these cases it is evident that little heat is developed because small currents pass through the apparatus. The measurements with highly conducting solutions (for example, 0.1 N hydrochloric acid or 0.1 N nitric acid) can be explained in the same way. Since the resistance is relatively low, less heat is generated. However, this latter case is complicated by the fact that the solution is followed by an unusually dilute indicator solution (0.03 to 0.05 N) in which, due to the high potential gradient, the heating effect must be unusually large. However, this hotter solution is on top rather than on the bottom in contrast to the experiments described in Section 4, so that the small difference of temperature apparently increases the stability of the system rather than otherwise.

For use with solutions above 0.1 N we have constructed a cell with a thin-walled measuring tube with an internal diameter of only 2 mm. At the same rate of motion of the boundary this smaller tube requires only one-ninth of the current used in the 6 mm. tube, whereas the surface exposed is one-third. A distinct increase of the range of indicator concentrations yielding adjustment has been observed, as would be expected. However, other factors not yet clearly understood affect adjustment. We expect to make this matter the subject of future investigations.

7. THE TRANSFERENCE NUMBER OF 0.05 N SILVER NITRATE SOLUTION

The data we have accumulated on transference numbers will be published in an article dealing with the question of the ionization of strong electrolytes. It may be of interest, however, to give an example of the precision attainable, under favorable circumstances, by the moving-boundary method. A plot of the data on 0.05 N silver nitrate solution is given in Fig. 6, in which the observed transference numbers are plotted as ordinates and the indicator concentrations as abscissas. The concentration of the indicator solution

(lithium nitrate) varied from 0.0385 N to 0.0410 N. It will be seen that adjustment was attained through nearly all of this range within about 0.02%. A slight drop in the transference number can be observed in the region of the dilute indicator solutions. Experiments were not carried out at higher values of the indicator solution than those shown, but from analogy to other cases studied the curve would eventually ascend. The value for the transference number obtained, 0.4664 at 25°, is lower than published values by the Hittorf method, which are nearer 0.470.

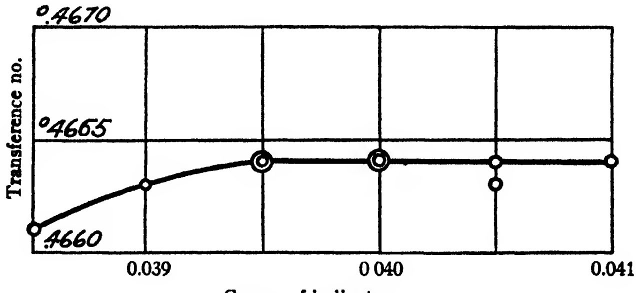

Fig. 6.

SUMMARY

Improvements in the moving-boundary apparatus for determining transference numbers, leading to greater accuracy and convenience in the use of the method, are described.

The measurements have been found to be much influenced by vibrations, particularly where there is much difference in density of the solutions at the boundary. Our method for eliminating this difficulty is outlined.

Experiments are described which indicate that boundaries must be descending during the determinations. Ascending boundaries give rise to heat effects which cause mixing.

The conditions governing the visibility of boundaries are outlined.

Some of the factors governing "adjustment," that is, the dependence of the motion of the boundary on the indicator concentration, are outlined.

The precision of the method now attainable is shown by measurements of the transference number of 0.05 N silver nitrate solution.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1927, Vol. x, No. 6, pp. 883–892]

THE KINETICS OF OSMOSIS.

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(Accepted for publication, April 27, 1927.)

The equilibrium conditions in osmotic pressure experiments have been thoroughly investigated both theoretically and experimentally. The kinetics of the process, however, has received little attention although the general theory is known. The process of osmosis is essentially one of diffusion of the solvent into the solution and so should follow in general the diffusion laws. In ordinary diffusion experiments, however, such as the solution of a solid or the diffusion of salt through a vessel of water, the solute is the component which moves while in osmosis it is the solvent. In the former case, since the number of solute molecules is ordinarily only a small fraction of the total number, the total number of molecules remains practically constant, while in osmosis, the total number of molecules in the solution changes during the experiment. It might be expected therefore that the equation for osmosis would differ slightly from that of diffusion of the solute since the terms containing the total number of molecules, *i.e.* the volume, which are constant in the ordinary diffusion formula are now variables. As will be seen this is the case experimentally.

The desired relation may be derived in a number of ways, but the following derivation, although not mathematically rigorous, appears to the writer to be the simplest.

Assume the solution separated from the solvent by a membrane permeable only for the solvent, as shown in Fig. 1. The mole fraction of the solute is assumed small and the solution is assumed to obey the laws of ideal solutions Solvent will pass through the membrane from the pure solvent into the solution. The volume and hence the hydrostatic pressure on the solution will be increased and the process

will stop when the hydrostatic pressure equals the osmotic pressure. It is desired to know the quantity of solvent which passes through the membrane at any time.

According to the general law of diffusion (or flow) the rate of flow per unit area is proportional to the pressure gradient, or, in this case, to the pressure divided by the resistance; or

$$\frac{dv}{dt} \sim \frac{P}{R_1},$$

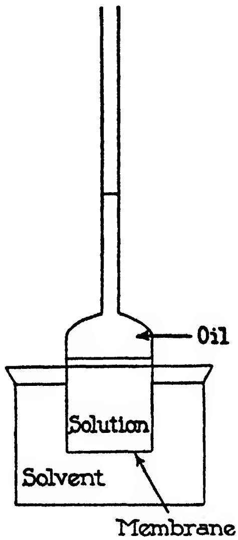

FIG. 1. Apparatus for the determination of the rate of osmosis.

in which v is the volume, t the time, P the pressure, and R the resistance offered to the flow.

In order to integrate this equation the variable terms must be expressed as functions of v or t. The pressure is evidently equal to the osmotic pressure, which tends to force the water in, less the hydrostatic pressure, which tends to force it out.

$$P = OP - HP.$$

The osmotic pressure is defined by the equation,[1]

$$OP = \frac{RT}{V_m}(-\ ln(I - x))$$

which for dilute solutions reduces to

$$OP = \frac{RTx}{V_m} \tag{1}$$

where R is the gas constant, T the absolute temperature, x the mole fraction of the solute, and V_m the molal volume of the solvent in the solution.[2] Assume there are g gm. of solute of molecular weight M dissolved in V cc. of water. The mole fraction of the solute, then, will be the moles of solute divided by the total moles, or

$$x = \frac{\frac{g}{M}}{\frac{V}{V_m} + \frac{g}{M}}.$$

Since $\frac{g}{M}$ is assumed small in comparison to $\frac{V}{V_m}$, the equation may be written $x = \frac{gV_m}{MV}$. Substituting this value of x in (1),

$$OP = \frac{RT}{V_m} \cdot \frac{gV_m}{MV},$$

[1] *Cf.*, for instance, Washburn, E. W., Physical chemistry, New York, 1st edition, 1915, 155.

[2] Since we are interested only in the *amount* of water that diffuses into the system, *i.e.*, the amount that passes through the plane at the outside surface of the membrane, it is not necessary to consider the pressure gradient within the solution. The pressure may therefore be assumed proportional to the average pressure, *i.e.*, to the pressure that would exist if the solution were homogeneous. That this is so may be seen from the fact that the pressure gradient would depend on the diffusion coefficient, which does not affect the form of the equation but only the value of the constant. Mathematical proof of this statement may be found in the fact that if the equation is solved according to the general form of Fouriers theorem, which takes into account the pressure gradient and gives the amount of water which passes through a plane at any distance y, from the surface of the membrane, and y is then made equal to zero, the equation reduces to the same form as the integral of (1). *Cf.* Mellor, J. W., Higher mathematics for students of chemistry and physics, New York, 4th edition, 1913, 488.

or, since in any one experiment R, T, M and also g are all constant and may be combined into one constant P_o,

$$OP = \frac{P_o}{V}. \tag{2}$$

The hydrostatic pressure will be equal to the initial pressure, n', plus the additional pressure caused by the rise of the solution in the capillary. If 1 cc. increase in volume causes the liquid to rise K' mm., and f is the relative specific gravity of the liquid compared to mercury, then the hydrostatic pressure at any time expressed in mm. of mercury will be equal to $fn' + fK'(v - v_o)$, or $HP = n + K(v - v_o)$, where v is the volume at any time and v_o is the initial volume; n is the initial pressure expressed as mm. mercury; and K is the increase in pressure per cc. increase in volume, expressed also as mm. mercury.

Evaluation of R_1.— R_1 may be expressed in different ways depending on the mechanism assumed for the passage of water through the membrane. If the water is assumed to dissolve in the membrane and so pass by diffusion, R_1 is a function of the diffusion coefficient. If the water is assumed to flow through capillaries, then R_1 is a function of the size and number of the capillaries.

1. The Water Dissolves in the Membrane.

The thickness of the layer of solution for a cylindrical vessel with the membrane at one end will be $\frac{v}{\pi r^2}$, where r is the radius of the cylinder. If the thickness of the membrane is h then the total average distance the water has to diffuse will be $h + \frac{v}{2\pi r^2}$, and the total resistance offered to its flow per unit of area will be the distance times the specific resistance; or if R_m is the resistance offered by unit thickness of collodion and R_w the resistance offered by unit thickness of the solution, the total resistance $R_1 = hR_m + \frac{v}{2\pi r^2} R_w$. If the membrane is of such a nature therefore that the solvent can diffuse through it as rapidly or nearly so as through the solution, it is evident that the resistance offered by the membrane may be neglected, since the

distance passed through in the membrane is very small compared to the total distance, and $R = \frac{v}{2\pi r^2} R_w$. In the case of collodion membranes and most other artificial membranes the resistance offered by the membrane is enormously greater than that offered by the solution, so that the term representing the resistance of the solution may be neglected and $R = hR_m$. Since the diffusion coefficient is the reciprocal of the specific resistance, $R = \frac{h}{C}$ where C is the diffusion coefficient of the solvent in the membrane.

2. The Water Flows through Capillaries in the Membrane.

In the evaluation of the resistance given above the solvent was assumed to diffuse through the membrane in the same way as through the solution and the increase in resistance was ascribed to the difference in the rate of diffusion of the solvent molecules in the membrane and in the solution There is some reason to believe, however, that collodion membranes at any rate may be considered as consisting of pores in a solid and that the water passes only through the pores. From this point of view the resistance offered by the membrane will be determined by Poiseuille's law.[8] The resistance offered by the solution can again be neglected. If there are p pores of radius r_1 per unit of surface, and they are assumed to be the same length as the thickness of the membrane, the quantity of water that will pass under unit pressure according to Poiseuille's law will be proportional to $\frac{pr_1^4}{h\eta}$, η being the viscosity, and the resistance offered to the passage of the water will be the reciprocal of this or $\frac{h\eta}{pr_1^4}$. Since for any one solvent and membrane η, p and r_1 are constant the resistance will be $\frac{h}{C}$ as before.

In either case, then, the total amount of water passing through will be proportional to $\frac{SC}{h}$ where S is the total surface of the membrane.

[8] *Cf.* Hitchcock, D. I , *J. Gen. Physiol* , 1925–27, viii, 71.

Substituting these values of R and P, equation (1) becomes

$$\frac{dv}{dt} = \frac{CS}{h}\left[\frac{P_o}{v} - [n + K(v - v_o)]\right]. \tag{3}$$

C is the quantity of solvent that will pass through a unit area of membrane of unit thickness in unit time under unit pressure. Changes in the value of C are due then either to changes in the rate of diffusion in the membrane or to changes in the pore size or number, or the viscosity of the solvent, depending on which mechanism is assumed for the passage of the solvent through the membrane.

If there is no hydrostatic pressure on the solution at the beginning of the experiment the equation in this form predicts that the quantity of solvent passing through the membrane in the first few minutes will be proportional to the osmotic pressure of the solution This relation has been shown to be true by a number of workers and has been used to measure the pressure in cases where the equilibrium value could not be obtained.[4]

At equilibrium no solvent passes through the membrane, *i.e.* $\frac{dv}{dt} = 0$, so that

$$\frac{P_o}{v_e} = n + K(v_e - v_o), \tag{4}$$

or

$$Kv_o = n + Kv_e - \frac{P_o}{v_e},$$

where v_e is the volume of solution at equilibrium.

Equation (3) may be integrated in a number of forms depending on which constants are used. Mathematically the simplest expression is obtained in terms of P_o, v_e and K. In order to obtain the equation in these terms the value of Kv_o, from equation (4), is substituted in equation (3). Collecting terms and simplifying, the equation becomes $\frac{dv}{dt} = \frac{CSP_o}{hv_e}\frac{(1 + bv)(v_e - v)}{v}$, where $b = \frac{Kv_e}{P_o}$, which, on integration, gives

$$C = \frac{2.3\ hv_e}{(1 + bv_e)SP_ot}\left(v_e \log \frac{v_e - v_o}{v_e - v} - \frac{1}{b} \log \frac{1 + bv}{1 + bv_o}\right); \tag{5}$$

[4] *Cf.* Findlay, A., Osmotic pressure, London and New York, 2nd edition, 1919.

or if $K = 0$, that is when the experiment is so arranged that the hydrostatic pressure is constant,

$$= \frac{hv_e}{SP_o t}\left(v_o - v + 2.3\, v_e \log \frac{v_e - v_o}{v_e - v}\right). \qquad (6)$$

TABLE I.

Rate of Osmosis 30°C.

Experiment 1.

$v_o = 3.0$ $P_0 = 52.5$ $K = 2.28$ $n = 1.1$ $v_e = 6.2$ $b = .27$
$S = 10$ sq. cm.

T		$K_m \times 10^4$	$\frac{C}{k} \times 10^4$
hrs.	*cc*		
0	3.0		
24	3 70	47 5	2.27
48	4 20	44.0	2.19
96	4.85	38 0	1 96
192	5 60	36 0	1 98

Experiment 2

3.2 cc. a "soluble" gelatin in thistle tube closed with collodion membrane.
$v_o = 3.2$ $P_o = 288$ $K = 0$ $n = 50$ $v_e = 5.85$ $S = 4.5$ sq. cm.

T	v	$K_m \times 10^4$	$\frac{C}{k} \times 10^4$
hrs.	*cc*		
0	3 2		
24	3 75	42.5	1 53
48	4 15	40.0	1 53
96	4 72	38 7	1 62
192	5 22	32 0	1 51
300	5 85		

This condition would also be true if the solvent outside the membrane were replaced by a large volume of solution having a lower osmotic pressure than that of the solution inside. The osmotic pressure of the outside solution would enter into the equation in the same way as does the initial hydrostatic pressure, n, in equation (3).

A number of experiments were performed to test the accuracy of these equations, and they were found to hold within the experimental error.

Table I and Fig. 2 give the results of two such experiments. In the first experiment solutions of egg albumin in M$(NH_4)_2SO_4$ were placed in a rocking osmometer,[5] with the same concentration of ammonium sulfate outside, and left until equilibrium was established.

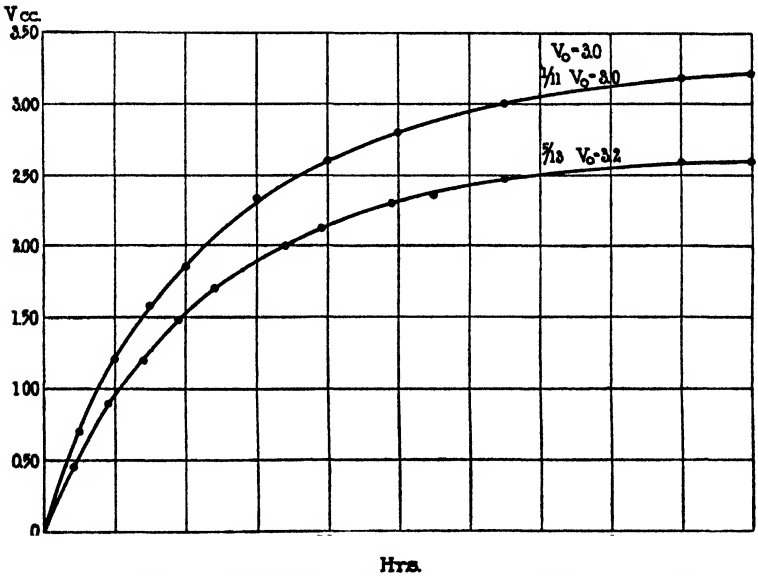

FIG. 2. The rate of osmosis of protein solutions in collodion membranes.

3 cc. of the solution was then placed in a tube closed with a membrane, the upper part of the tube filled with oil and the membrane immersed in the solution of ammonium sulfate with which the albumin solution had previously been in equilibrium. The pressure in the manometer was set at the equilibrium value and the system left for 2 days so that the permeability of the membrane might become constant. The manometer level was then lowered and the rise of the

[5] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 351; also 1926–27, x, 161.

oil in the manometer noted. The experiment was carried on in a water bath at 30°C. In the presence of this concentration of ammonium sulfate the osmotic pressure of albumin is nearly proportional to its concentration, so that it may be assumed to obey the ideal solution law. The ammonium sulfate also prevents bacterial growth. The second experiment was done in the same way except that a solution of "soluble" gelatin[5] was used. In this case the manometer tube was bent so as to be horizontal. There was therefore no change in pressure during the experiment and equation (6) should fit. The table shows in both cases that the monomolecular constant K_m given for comparison shows a regular decrease while the constants calculated by equations (5) and (6) do not vary outside of the experimental error. This was found to be the case in all of the experiments made. The monomolecular constant dropped slowly in every case.

In the first experiment the value of $\frac{C}{h}$ was found to be 2×10^{-4}. If the derivation given is correct this should be the cc. of water that will flow through 1 sq. cm. of the membrane in 1 hour under 1 mm. mercury pressure. At the end of the experiment the membrane was washed, filled with water and the rate of flow of water through it determined under 10 cm. mercury pressure. A value for the rate of flow of 1.5×10^{-4} cc. per hour per mm. mercury pressure was obtained, which agrees as well as could be expected with the figure calculated from the osmotic pressure experiment.

In this experiment the surface of the membrane is constant. In experiments with cells such as those of McCutcheon and Lucke[6] the surface increases during the experiment. If the water is assumed to diffuse through the membrane, the thickness of the membrane being constant, then $S \propto v^{\frac{2}{3}}$; or if the volume of the membrane remains constant $\frac{S}{h} \propto v^{s}$. If the water is supposed to flow through pores in the membrane and the increase in surface is due to enlarging the size of the pores, the thickness remaining constant, then $r^2 \propto S \propto v^{\frac{2}{3}}$ and $r^4 \propto v^{\frac{4}{3}}$ According to the first assumption the velocity should be very slightly slower than that predicted by the monomolecular formula and according to the second or third assumption it should be very

[6] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1925–26, ix, 697.

slightly faster. In either case the deviation from the monomolecular formula would be noticed only in very accurate experiments.

SUMMARY.

It is shown that by combining the osmotic pressure and rate of diffusion laws an equation can be derived for the kinetics of osmosis.

The equation has been found to agree with experiments on the rate of osmosis for egg albumin and gelatin solutions with collodion membranes.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1927, Vol. x, No. 6, pp. 893–904.]

THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

I. EQUILIBRIUM CONDITIONS.

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(Accepted for publication, April 27, 1927)

If a block of isoelectric gelatin is placed in water it will imbibe water and increase in volume. The writer and Kunitz (1) were able to measure the pressure with which this water was drawn into the block and found that it increases with the concentration of the gelatin. According to the phase rule the concentration (osmotic pressure) of a solution in the presence of the solid is independent of the amount of solid. It was suggested therefore that gelatin consisted of two or more fractions some of which were insoluble at low temperature while others were soluble and so could exert osmotic pressure. The swelling of isoelectric gelatin then becomes a process of osmosis just as Wilson (2) showed was the case for the swelling of gelatin in acid, except that in the case of isoelectric gelatin the osmotic pressure is not due to the ions of an electrolyte but to the presence of a soluble constituent of the gelatin itself. Water therefore enters the gelatin until the elastic pressure is equal to the osmotic pressure. The present paper is an attempt to apply this mechanism quantitatively to the swelling of gelatin.

The general behavior of gelatin when placed in water has been described by a number of investigators and in detail by the very complete experiments of Arisz (3). The more striking peculiarities may be briefly described as follows. In general the swelling increases with the temperature and with the concentration of gelatin. A block of gelatin concentrated by allowing the water to evaporate swells much more than a similar block made by allowing a solution of the same concentration to solidify. Thin films of gelatin reach a value which increases only slowly while large blocks do not give any indication of a maximum value but continue to swell until dissolved. At higher

temperatures there is less indication of an equilibrium value. If a block of gelatin is allowed to remain in water until it has stopped swelling and then is raised to a higher temperature in air for a short time under such conditions that there is no change in volume, it will swell rapidly when replaced in water at the first temperature. These peculiarities are similar to those of any substance when under an elastic strain, and can be readily accounted for at least qualitatively by the mechanism of swelling stated above. When the block is placed in water, water enters owing to the osmotic pressure of the solution in the block. The fibres of solid material are thereby forced apart and the force with which they attempt to return to their original position opposes the entrance of the water. At the same time the osmotic pressure is decreasing owing to dilution. When the elastic force equals this osmotic pressure the process stops. If the fibres have been stretched beyond their elastic limit, however, or if the force is applied for too long a period of time the fibres become fatigued and the elastic force is lessened so that more water enters. As is the case with any elastic body therefore a true equilibrium value is never reached. The osmotic pressure increases with the temperature and at the same time the amount of solid material decreases and also probably becomes less elastic so that swelling increases very rapidly as the temperature rises. In a thin film the whole mass becomes filled with water before the fibres become fatigued whereas in a large block the outside layers, which swell first, become fatigued and take up more water before the water has diffused into the inner layers. This "secondary swelling" therefore overlaps the primary so that in the case of large blocks there is no indication of a maximum value. This mechanism will be discussed more in detail under the kinetics of the swelling process. When a block which has swollen at a low temperature is raised to a higher temperature the elasticity is destroyed and it therefore swells again when replaced in water. When the gelatin is concentrated by evaporation it decreases in size and is therefore under an elastic strain just as when it swells except that in the case of loss of water the elastic force is in the same direction as the osmotic pressure instead of opposite to it. The block therefore swells more than a similar one which is not under elastic strain at this concentration.

Experimental Procedure.

In order to avoid the difficulty of working with very thin pieces of gelatin the gelatin was coated on glass rods about 0.2 by 15 cm. The rods were weighed, dipped in gelatin of the desired strength at a temperature of 40°C., weighed again in order to determine the amount of gelatin in the film and then placed in stoppered tubes containing a strip of wet filter paper in order to prevent loss of water by evaporation.[1] The tubes were then put in the cold room at 5°C. Arisz found that gelatin swells more if placed in water at a low temperature immediately after solidifying than if kept at the same temperature in air for a time before placing in water. After the 1st day no further change occurred. These experiments were repeated and confirmed except that a change was noted for the first 3 days. All the gelatin used in these experiments was therefore kept at 5° for 3 days before it was placed in water. If, as the writer assumes, solid gelatin contains a saturated solution of the material forming the network this is exactly the behavior expected since time is required to reach the equilibrium value. The experiment is analogous to that of Loeb (5) who found that the osmotic pressure of a gelatin solution cooled from 70° to 37° was higher at first than that of a solution raised from 10° to 37°, but later became the same. At the beginning of an experiment the rods were weighed again and then placed in water at 5° and weighed at intervals after wiping dry with soft filter paper. Since warming even for a short time destroys the elasticity, it is necessary to weigh at the same temperature as that at which the swelling occurred. Some

[1] Schroeder found that gelatin which was in apparent equilibrium with water lost weight when suspended in saturated water vapor at the same temperature and this has been considered as contrary to the second law of thermodynamics. As Bogue (4) has pointed out, however, the force of gravity is acting on the gelatin in the air but not in the water and if the gelatin is supposed to consist of a network of capillaries, this is sufficient to account for the observation. Suppose a fine capillary is dipped into water and after the water has risen in the tube, the tube is removed and suspended vertically just above the surface of the liquid. A drop will be formed at the bottom of the tube and since this drop has a convex surface its vapor pressure will be higher than that of the body of water. Water will evaporate from the drop until the surface of the water at the bottom of the capillary is no longer convex.

experiments were also made with slides dipped in gelatin and with thin slices of gelatin cut from a cylinder. Spheres of gelatin made by dropping liquid gelatin into cold toluene were also used.

For convenience in the calculation and presentation of the results they have been expressed in terms of gm. of water per gm. of gelatin.

Measurement of the Swelling Pressure.—The pressure with which the water tends to diffuse into the gelatin was measured by the writer and Kunitz (1). The measurements were extended to higher concentrations using the same method as described. The results of these

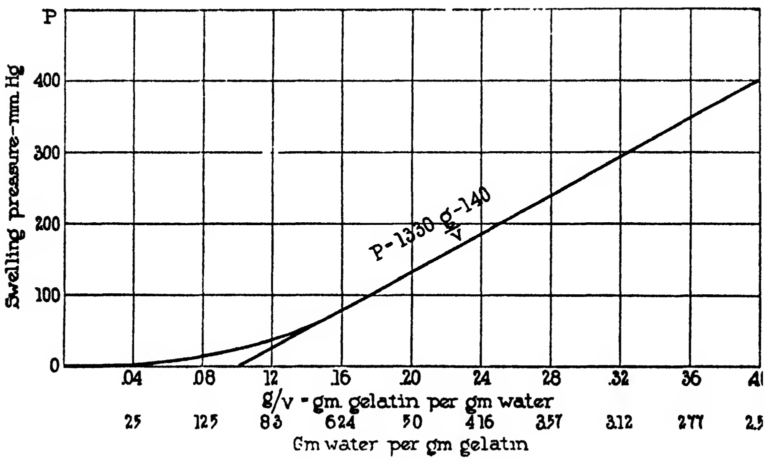

FIG. 1 Concentration-swelling pressure of gelatin at 10°.

measurements of the swelling pressure of isoelectric gelatin at 5° are shown in Fig. 1.

These pressures were obtained with gelatin that had been made up to the concentration noted while liquid and it seemed quite possible that a different pressure would be obtained when the concentration was changed by a swelling of the solid gelatin. In order to test this point a Chamberland filter was coated with collodion and then with gelatin containing 40 gm. per 100 gm. of water. The thimble was then placed in water for 12 hours at the end of which time the concentration was 30 gm. of gelatin per 100 gm. of water. The filter and

gelatin were then removed from the water, the inside filled with water and a manometer tube attached as previously described (1). At first no pressure was obtained as the swelling pressure was balanced by the elasticity of the gelatin. As the elasticity decreased with time, however, the pressure gradually rose and after 4 days remained constant at 255 mm.Hg pressure. This is the same, within the experimental error, as the value obtained with 30 per cent gelatin which had been prepared by adding water to the melted gelatin. The effect on

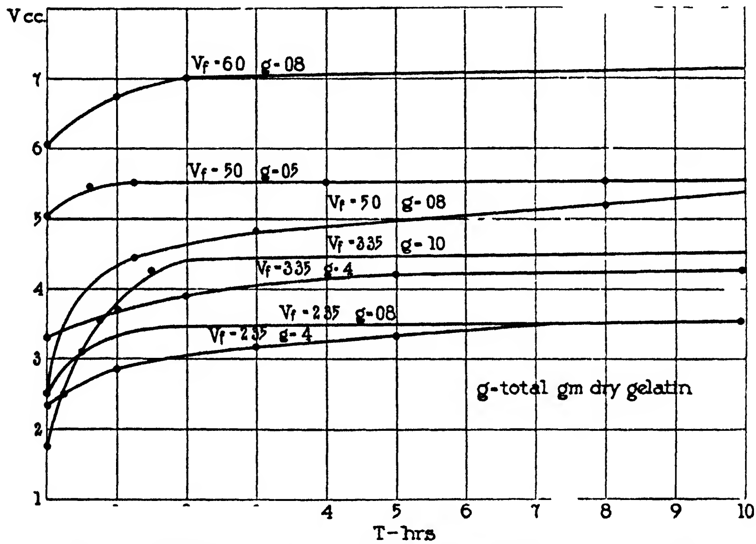

FIG 2 Swelling of various concentrations of gelatin in water at 5°.

the osmotic pressure caused by the addition of water is therefore the same whether the water is added to the solid or liquid gelatin.

The results of some typical swelling experiments are shown in Fig. 2. It is evident that at this temperature and with thin films of gelatin, a fairly constant maximum value is reached. The experiments also show that the final value for the swelling depends only on V_f, the concentration of the gelatin at the time it solidified and, within the limits of variation used in these experiments, is independent of the concentration of the gelatin when it is put in water.

Calculation of the Equilibrium Conditions.

According to the mechanism of swelling outlined above equilibrium is reached when the osmotic pressure equals the elastic pressure. The elastic pressure in this case takes the place of the hydrostatic pressure in the case of osmosis experiments discussed in a preceding paper (6). At equilibrium then

$$OP = EP \tag{1}$$

In order to use this relation both pressures must be expressed in terms of the volume of water. In ideal dilute solutions the osmotic pressure may be written $OP = P_o/V$ where P_o is a constant depending on the concentration and molecular weight of the solute, the molal volume of the solvent and the temperature, and V is the gm. solvent. It has been shown by Kunitz (7) that the osmotic pressure of gelatin solutions also obeys this law when the proper correction is made for the amount of solvent combined with the gelatin. The relation is rather complicated, however, and it is more convenient to use an empirical relation. It may be seen from Fig. 1, that at concentrations of between .1 and .4 gm. gelatin per gm. of water, the swelling pressure curve may be represented by the equation $P = \dfrac{1330g - 140v}{v}$ where g is the gm. gelatin and v the gm. water or $P = \dfrac{1330 - 140\,V}{V}$ where V is the gm. water per gm. gelatin.

The bulk modulus of an elastic body by definition is proportional to the elastic force divided by the difference between the volume when under no strain and the volume under the force applied. Or in terms of the original volume, it is the force required to increase the volume by an amount equal to the original volume.

$$K_e = \frac{EP\ V_f}{V - V_f} \quad \text{and} \quad EP = \frac{K_e\,(V - V_f)}{V_f}$$

where V_f is the volume when under no strain and K_e is the bulk modulus. Substituting these values of OP and EP in (1)

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e\,(V_e - V_f)}{V_f}$$

where V_e is the volume at equilibrium or

$$K_e = \frac{1330(1 - .105V_e)}{(V_e - V_f)} \frac{V_f}{V}$$

$$V_f = \frac{K_e V_e^2}{1330 + V_e(K_e - 140)}$$

$$V_e = \frac{(K_e - 140)V_f}{2\,K_e} \pm \sqrt{\frac{1330V_f}{K_e} + \left[\frac{(K_e - 140)V_f}{2\,K_e}\right]^2}$$

Ordinarily the total volume would be used in the calculation of the bulk modulus but since in the case of the osmotic pressure it is

TABLE I.

Swelling of Thin Layers of Gelatin on 2 mm. Glass Rods in H_2O at 5°.

V_f H_2O per gm gelatin when cast	V_e H_2O per gm gelatin after swelling		K_e	$\frac{K_e \times 77}{V}$
	Observed	Calculated $K_e = 500$		
2 35	3 5 ± 1	3 48	490	160
3 35	4 3 ± .2	4.40	550	126
5 0	5 7 ± 2	5 86	670	103
5 8	6 8 ± 3	6.56	350	46
Average			500	

better to use the volume of water rather than the total volume, the calculation is simplified by using the same value in the bulk modulus calculation. The use of the total volume instead of the volume of water would simply result in a different value for the bulk modulus. It may be noted that the volume of dry gelatin, however, cannot be used, as was done in an earlier paper since in that case the formula would predict that the swelling would depend only on the concentration of the block, which is not the case. The determining factor is the concentration at the time the solution solidified and not the concentration at the time it happened to be put in the water. In other words, as with any elastic body, it is necessary to define the change in volume as the change from the volume when under no elastic strain.

As will be shown later this "elastic volume" is the actual volume when the solution solidified.

A number of experiments were made as previously described and the average value of K_e calculated. The results are given in Table I. K_e is expressed in mm. mercury pressure The values of K_e vary somewhat but the equation is of such a form that the value of this constant is very sensitive to small differences in V_e. The value is really constant for the range of gelatin used as may be seen by the fact that the values of V_e calculated by assuming a constant value for K_e are equal, within the probable error of the measurement, to the observed values. If the value of K_e be defined as the force required to increase the volume by an amount equal to the volume of the dry gelatin in the block

TABLE II

Swelling of Gelatin in Various Forms.

$V_f = 5.8$				
	On glass rods	On slides	Spheres	Thin sheets cut from cylinder
V_e	6.8	6.7	7.5	7.4
K_e	350	370	130	140
$\frac{K_e \times .77}{V_f}$	46	50	13	14

rather than the volume of water, then the value given must be multiplied by the ratio of the volume of dry gelatin to that of the water. Since 1 gm. of gelatin occupies about .77 cc., the pressure required to increase the block by an amount equal to the volume of the dry gelatin contained in it will be $K_e\ .77/V_f$. This value decreases in proportion to the concentration of gelatin, as would be expected. The same relation had been found by Sheppard (8) to hold approximately for the relation of modulus of elasticity to the concentration of the gelatin.

Since these experiments were made with gelatin on glass the gelatin is prevented from elongating by the glass rod. It might be expected therefore that gelatin alone would swell more. This is the case as shown by Table II which gives the equilibrium concentration for

gelatin on slides, in the form of spheres or as thin pieces cut from a cylinder. It may also be noted that the value for the bulk modulus calculated on the basis of the volume of the dry gelatin agrees with that previously calculated indirectly from separate swelling and osmotic pressure experiments (9).

It has been assumed in these calculations that the gelatin when solidified is under no elastic strain and that this concentration is therefore the determining one for the swelling. As a corollary gelatin, the concentration of which has been changed after solidifying, is under elastic strain. It might be expected then that gelatin under these

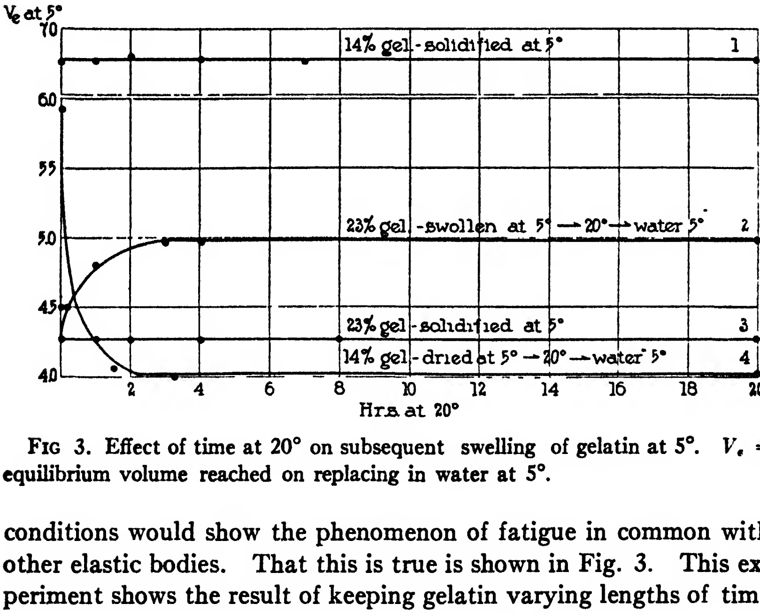

FIG 3. Effect of time at 20° on subsequent swelling of gelatin at 5°. V_e = equilibrium volume reached on replacing in water at 5°.

conditions would show the phenomenon of fatigue in common with other elastic bodies. That this is true is shown in Fig. 3. This experiment shows the result of keeping gelatin varying lengths of time at 20° after having been swollen or dried while solid. Curves 1 and 3 show that gelatin which has been allowed to solidify at 5° undergoes no change when kept in air at 20°. The swelling which occurs on replacing in water at 5° is the same whether or not the gelatin has been kept at 20°. Gelatin which has swollen at 5° before being placed at 20°, swells further on replacing at 5°, the amount of swelling depending upon the length of time the gelatin had been at 20°.

After the first 2 hours at 20° further exposure to this temperature causes no further change in the behavior of the gelatin. The swelling now is the same as that of freshly solidified gelatin of the same concentration. Gelatin which has been partially dried shows the same behavior in that it swells much less after exposure to 20° and the swelling soon reaches a constant value. This loss of elasticity occurs very rapidly at 20° but is much slower at 5°. This is shown by Fig. 4. In this experiment, three series of rods coated with 30 per cent gelatin were allowed to swell to the equilibrium value at 5°. All but two

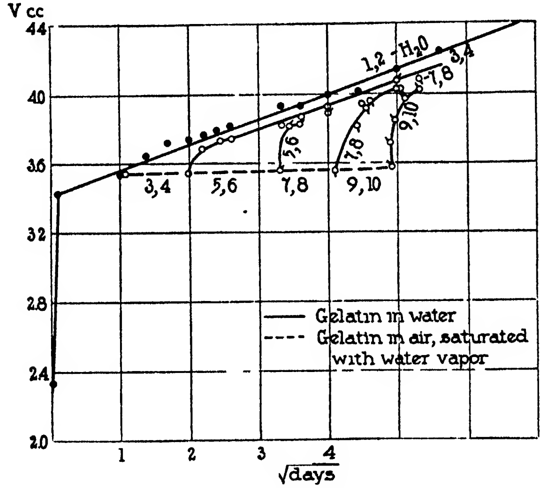

FIG. 4. Effect of time at 5° in air, after swelling, on subsequent swelling when the gelatin is replaced in water.

were then removed and placed in air at 5°. They were then returned to the water after varying periods of time. In the meantime the two rods which had been left in water continued the slow secondary swelling. On returning the other rods to water they swelled rapidly, the more so the longer the time since the beginning of the experiment, and very rapidly approached the value of those that had been in the water continuously. This shows that the secondary swelling is due to the loss of elasticity and that it is continually going on. As stated before a large block will therefore not show any maximum value since the

outside layers become fatigued and take in additional water before the internal layers have reached the equilibrium value. The same process will occur much more rapidly at a higher temperature so that even small blocks show no maximum. This secondary swelling will evidently continue until all the water has been taken up. If sufficient water is present a solution of gelatin will eventually result.

It may be noted that the secondary swelling in Fig. 4 is proportional to the square root of the time. The significance of this will be discussed under the kinetics of the process.

SUMMARY.

The swelling of isoelectric gelatin in water has been found to be in agreement with the following assumptions.

Gelatin consists of a network of insoluble material containing a solution of a more soluble substance. Water therefore enters owing to the osmotic pressure of the soluble material and thereby puts the network under elastic strain. The process continues until the elastic force is equal to the osmotic pressure. If the temperature is raised or the blocks of gelatin remain swollen over a period of time, the network loses its elasticity and more water enters. In large blocks this secondary swelling overlaps the initial process and so no maximum can be observed.

The swelling of small blocks or films of isoelectric gelatin containing from .14 to .4 gm. of dry gelatin per gm. of water is defined by the equation

$$V_e = \frac{(K_e - 140)}{2\,K_e}\,V_f \pm \sqrt{\frac{1330\,V_f}{K_e} + \left[\frac{K_e - 140)\,V_f}{2\,K_e}\right]^2}$$

in which K_e = the bulk modulus $= \dfrac{PV_f}{(V_e - V_f)} = \dfrac{1330(1 - .105\,K_e)}{(V_e - V_f)}\,\dfrac{V_f}{V_e}$.

V_e = gm. water per gm. gelatin at equilibrium; V_f = gm. water per gm. gelatin when the gelatin solidified.

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[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1927, Vol. x, No. 6, pp. 905–926.]

THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

II. KINETICS.

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(Accepted for publication, April 27, 1927.)

Measurements of the osmotic pressure and of the swelling of gelatin in salt solutions and of the swelling pressure of gelatin have led the writers[1] to assume that gelatin was a mixture of two substances or groups of substances, one of which is soluble and the other insoluble. Solid gelatin was therefore pictured as a network of the insoluble material holding a solution of the soluble protein in its meshes. The osmotic pressure of this soluble material was assumed to be the force which caused the block to swell. According to this mechanism the swelling of gelatin should be a special case of diffusion and should be fundamentally similar to osmosis. The peculiarity of swelling lies in the fact that the block of gelatin is both membrane and solution. Swelling differs from osmosis then in that the thickness of the membrane, *i.e.* the gelatin itself, increases during the course of the experiment, and the osmotic pressure is opposed by the elasticity of the gelatin rather than by the hydrostatic pressure. It might be expected therefore that the equation for the kinetics of the process while similar to that for osmosis would differ from it in some respects. This has been found to be the case.

As pointed out in the discussion of the kinetics of osmosis, the equation is of the same form whether the water is assumed to dissolve in and diffuse through the membrane or whether it is assumed to flow through capillary pores in the membrane. The only difference lies in the physical significance of the constants. There is some evidence that the flow of water through gelatin is also through pores and since this mechanism may be more easily analysed, it has been assumed in the present paper.

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

Formulation of the Equation.

According to Poiseuille's law the rate of flow of water through fine capillaries is proportional to the pressure times the fourth power of the radius and inversely proportional to the length of the capillary and the viscosity of the liquid. If the gelatin is assumed to consist of n pores per unit surface, then the total rate of flow of the water will be given by the expression

$$\frac{dv}{dt} = \frac{C' r^4 n S P}{\eta h} \qquad (1)$$

in which r is the radius of the pores, P the pressure, S the surface of the gelatin, h the length of the capillaries which is assumed to be the thickness of the gelatin, and η the viscosity of water. In order to use this expression the variable terms must be expressed as functions of v or t. In general, since the volume changes, the surface and thickness will also change. In the case of thin films of gelatin on glass, however, the surface may be assumed constant and the thickness therefore is equal to the volume divided by the surface, or $h = v/S$. The average distance traversed by the water in the case of swelling is half the thickness, so that $h/2$ must be used in place of h in equation (1).

Evaluation of P.—Since we are interested only in the amount of water which passes the outside surface of the gelatin it is not necessary to consider the pressure gradient in the gelatin, and the pressure may be assumed[2] equal to the average pressure. The pressure driving the water into the gelatin will be the difference between the swelling (osmotic) pressure which tends to cause the water to flow in and the elastic pressure of the gelatin which tends to force it out. Equilibrium is attained when these two pressures are equal. It was shown in the previous paper[3] that the swelling pressure at 5°C. from 0.14 to 0.4 gm. gelatin per gm. of water could be represented by the empirical formula

$$OP = \frac{1330 - 140V}{\text{[illegible]}},$$

[2] The validity of this assumption is discussed in the preceding paper, Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 883.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 893.

where V is the volume of water per gm. of gelatin, and the elastic pressure by

$$EP = \frac{K_e(V - V_f)}{V_f}$$

where K_e is the bulk modulus and V_f is the volume when under no strain. Therefore

$$P = \frac{1330 - 140V}{V} - \frac{K_e(V - V_f)}{V_f}. \tag{2}$$

At equilibrium these two pressures are equal, *i.e.*

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e(V_e - V_f)}{V_f} \quad \text{or} \quad K_e = \frac{1330(1 - .105V_e)\ V_f}{(V_e - V_f)\quad V_e}. \tag{3}$$

Evaluation of the Radius.—In the case of most membranes the radius of the pores would be constant, but in the case of gelatin this is probably not the case. If a block of gelatin is considered in any way analogous to a mass of separate fine particles of gelatin, then it is evident that the size of the pores which corresponds to the space between the particles will decrease rapidly as the particles swell. The exact function cannot be foretold as there is not sufficient evidence in regard to the actual structure of the gelatin. The simplest assumption, however, is that the radius of the pores between the particles would decrease as the radius of the particles increased or, approximately, $r^4 = c/V$. It will be assumed that the number of pores is constant. The size of the pores will also vary with the original concentration of the gelatin, but since this is constant for any one experiment it need not be taken into account here.

It is much more convenient to express the results in terms of the volume of water per gm. of gelatin rather than as the total volume. If g is the gm. of dry gelatin and V the volume of water per gm., then Vg equals the total volume of water, v, and $dv = gdV$.

As in the case of osmotic pressure, the equation may be solved in various ways depending upon which constants are used. The simplest expression is obtained in terms of K_e[4] the bulk modulus, V_e the equi-

[4] Since V_e may be expressed in terms of K_e and V_f it would theoretically be possible to express the equation in terms of V_f and K_e alone. Actually, however, this can only be done if a number of swelling curves are made with similar

librium volume, and V_f the volume when under no strain. In order to obtain the equation in this form equations (1) and (3) are added and the resulting equation solved for P.

Substituting these values of h, P, r^4 and v in equation (1) and simplifying and collecting the constant terms, equation (1) becomes

$$\frac{dV}{dt} = \frac{2CS^2\,1330(bV + V_f)\,(V_e - V)}{V^2 g^2 V_e V_f},$$

where $b = K_e V_e/1330$; on integration, this becomes

$$C = \frac{g^2 V_e V_f}{2 \times 1330\, S^2 t}\left[\frac{V_o^2 - V^2}{2b} + \frac{(bV_e - V_f)\,(V_o - V)}{b^2} + \frac{2.3\, V_e^2}{(bV_e + V_f)}\right.$$
$$\left.\log\left(\frac{V_e - V_o}{V_e - V}\right) - \frac{2.3\, V_f^2}{b^2(bV_e + V_f)}\log\left(\frac{V_f + bV}{V_f + bV_o}\right)\right] \quad (4)$$

In these experiments the last term is negligible in most cases. If K_e, the bulk modulus, is small, however, the last term becomes significant. C is the rate of flow of water under unit pressure through unit thickness and unit area of gelatin of concentration V_f. In the units used in these experiments it is cc. per hour, per sq. cm. surface, per cm. thickness per mm. mercury pressure.

In order to test this equation a number of experiments were performed with films of gelatin of various concentrations on glass slides. The gelatin was heated to 40°C., the slides weighed and then dipped in the gelatin. They were then cooled and weighed again and placed at 5°C. in stoppered tubes with wet filter paper. It was shown in the preceding paper[3] that the pressure changes for the first 3 days and then remains constant, so that in order to obtain reproducible results and also since the pressure-concentration curve is based on the value of the equilibrium pressure, the slides were usually allowed to remain at this temperature for 3 days before immersing in water. They were

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films and the results averaged before applying the equation. This is due to the fact that the value of V_e, and hence of K_e, varies slightly in individual experiments and the value of C is very sensitive to small differences in the value of V_e, especially near the end of the curve. It is therefore necessary in the calculation of individual curves such as those given to use the value of K_e and V_e determined from the particular experiment in question.

TABLE I.

Swelling of Films of Gelatin on Glass.

Experiment	S	g	V_f	V_e	t	V	$C \times 10^6$
	sq. cm.	gm.	cc	cc.	hrs	cc.	
2/2 *a*	25	.05	6 1	6.9	0	6 12	
					05	6 50	1.15
					.10	6 65	1 00
					.20	6.77	.80
1/13 *a*	25	.045	6 0	6.75	0	6 0	
					.05	6.32	.63
					.10	6 48	.61
					.20	6 66	.49
2/2 *b*	25	.12	6 1	6 9	0	6 12	
					32	6 50	1 05
					55	6 65	1 03
					1 00	6 75	.85
c	25	.14	6.0	6 75	0	6 0	
					2	6 27	1 22
					4	6 45	1 30
					.8	6 62	1 30
1/24 *a*	25	.063	5.9	6 75	0	2 45	
					10	5 0	40
					.20	5 82	.48
					.40	6 42	.56
1/20	27	.16	5 0	5 4	0	5 0	
					.5	5 15	.140
					1 0	5 225	.123
					1.5	5.275	.120
1/24 *b*	27	.052	3 80	5 0	0	1 55	
					.05	3 3	.120
					.10	4 0	.118
					.20	4 45	.119
1/13 *b*	25	.10	3 35	4 20	0	3 35	
					.05	3 70	.360
					.10	3 82	.284
					.20	3.95	.235
12/29	42	.31	2 35	3.4	0	2 23	
					.125	2.62	.125
					.25	2.82	.122
					.50	3.0	.110

then weighed again and placed in water at 5°C. They were removed at intervals, the excess water removed with soft filter paper and weighed in air at the same temperature. This is necessary since even slight warming destroys the elasticity and greatly increases the swell-

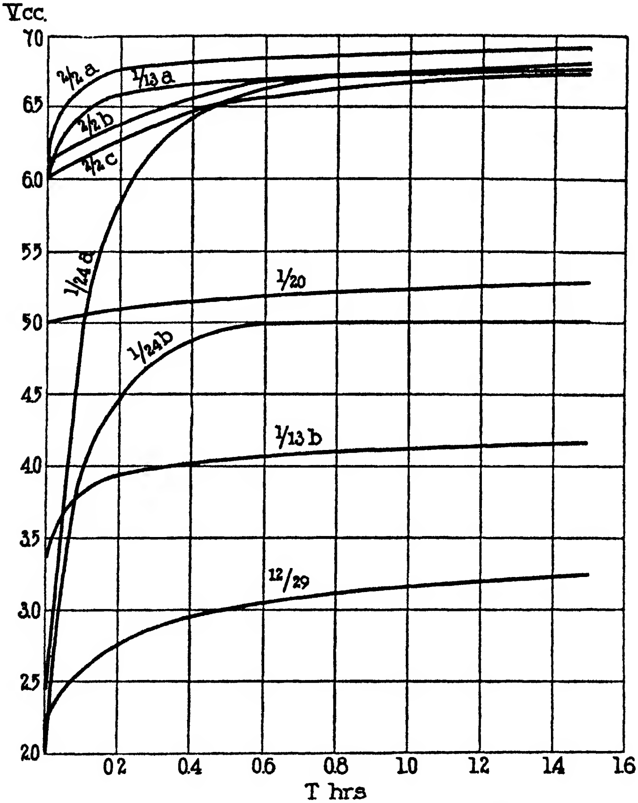

FIG. 1. Swelling films of gelatin on glass.

ing. It is also important to be sure that no change in weight has occurred before the slides and gelatin are weighed for the first time, since in the calculations it is assumed that the gelatin is of the same concentration as the solution of gelatin in which they were dipped. In some experiments the gelatin was allowed to dry partially at 5°

before placing in water. In this case the gelatin swells much more than the same concentration of gelatin which had been solidified at that concentration. It was shown in the preceding paper[3] that the equilibrium reached could be calculated by assuming that the concen-

TABLE II.

Swelling of Gelatin Spheres and Plates.

13.9 per cent gelatin heated to 40° and allowed to drop slowly into 200 cc. cold toluene in 250 cc. graduate. 20 spheres used for experiment.

13.9 per cent gelatin solidified in test-tubes. 5°, 3 days. Gelatin removed by warming and sections cut.

g	V_f	V_e	t	V	$C \times 10^{\text{[illegible]}}$
Spheres					
gm/sphere	*cc*	*cc*	*hrs*	*cc.*	
002	6.4	7 7	0	6 4	
			1 0	6 8	.85
			2 0	7 06	84
			4 0	7 40	.92
.005	6 4	7 7	0	6.4	
			1 5	6.8	1 02
			2.8	7 06	1.10
			7 0	7.40	0.97
Sections cut from cylinder 2 0 cm. diameter					
.037	6 3	7.5	0	6 2	
			.5	6 9	2 00
			1.0	7 13	1 75
			2 0	7 35	1.65
.10	6.3	7.5	0	6 2	
			3.0	6 9	2.45
			5 75	7.13	2 20
			11.0	7.35	2.15

tration when under no strain, V_f, was the concentration when the gelatin solidified. That is, the gelatin will swell to the same final value as though it had not been dried. This is strictly true only within certain limits and provided too long a time has not elapsed after drying,

as otherwise the gelatin becomes fatigued and V_f assumes a different value.

The results of some of these experiments are shown in Table I and in Fig. 1. The value of C varies slightly, but as a whole the equation appears to fit the experiment in a satisfactory way. The value of C is greater the greater the value of V_f, that is the more dilute the gelatin. This means that water flows more easily through dilute gelatin, which is a reasonable result. It can be confirmed, as will be shown

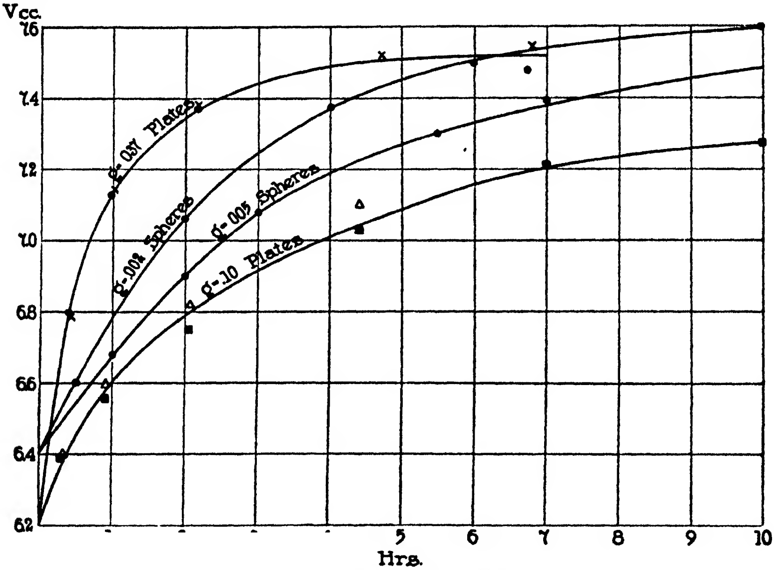

FIG. 2. Swelling of gelatin plates and spheres.

below, by direct measurement. In spite of the precautions noted some of the experiments show an anomalous course in that the gelatin swells either more or less than others of the same series. Experiment 1/20 is an example. The swelling in this case was very much less than would be expected from the average bulk modulus, and therefore the value of C although reasonably constant for this one experiment does not fit in with the other experiments. If the gelatin is placed in water immediately after solidifying, the opposite result is obtained, the gelatin swelling much more. This was ascribed to the fact that the pres-

sure is too high since it is assumed that the block is a saturated solution of one of the constituents and it requires time for the equilibrium concentration to be reached. In such cases also it was found that the value of C would be constant for that particular experiment but would differ from the other values.

Plates of Gelatin.—In the case of thin plates cut transversely from a cylinder of gelatin conditions are approximately the same as in the case just considered, since here also the greatest change in dimension is an increase in thickness and the surface may be considered constant. The results of some experiments with such plates are shown in Fig. 2 and Table II. The results have been calculated by formula (4) as before.

Spheres.—In the case of spheres the thickness instead of increasing directly as the volume increases only as the cube root of this value. The average distance which the water has to move is 1/6 the radius, which is 1/2 the ratio of volume to surface or

$$h/2 = r/6.$$

In these experiments the volume increases less than 20 per cent, so that the cube root of the volume may be considered constant for any one sphere and h assumed equal to $r_o/3$. This assumption is made in order to avoid the mathematical difficulties attendant on the integration of the fractional power of V. The surface also increases, but since the significant factor is the total number of pores and since the number presumably remains the same during any one experiment, S will also be considered a constant for any one experiment. In any case the effect of considering S variable would be within the limit of experimental error. Therefore $S = 4\pi r_o^2$ and $S/h = 12\pi r_o$, or $24\sqrt[3]{v_o} = 24\sqrt[3]{V_o g}$, approximately.

Substituting this value for S/h, equation (1) becomes

$$\frac{dV}{dt} = \frac{2 \times 1330 \times 24C\sqrt[3]{V_o g} \times (bV + V_f)(V_e - V)}{g\, V^2 V_e\, V_f};$$

or on integration:

$$C = \frac{g^{\frac{1}{3}}\, V_f V_e}{2 \times 1330 \times 24\sqrt[3]{V_o}\, t}\left[\frac{V_e - V}{b} + \frac{2.3 V_e^2}{bV_e + V_f}\log\frac{V_e - V_o}{V_e - V} + \frac{2.3 V_f^2}{b^2(bV_e + V_f)}\log\frac{bV + V_f}{bV_o + V_f}\right] \tag{5}$$

The last term is again negligible except when K_e, the bulk modulus, is small, *i.e.* when b is small.

TABLE III.

Swelling of Gelatin Cylinders.

Linen thread 15 cm. long dipped into melted gelatin.

$l = 15$ cm.

Experiment	g	V_f	V_e	t	V	$C \times 10^4$
	gm.	cc.	cc.	hrs.	cc	
11/1	.08	5.80	6 90	0	5.8	
				.5	6.15	1.16
				1.0	6.37	1.15
				2.0	6.67	1 35
	.15	5.80	6 90	0	5.8	
				1.0	6 17	1.23
				2.0	6 40	1.22
				4.0	6.60	1 05
10/7	.25	6 0	7.8	0	6 0	
				2	6 5	1.40
				6	6.97	1.22
				16	7 50	1 18
11/2	.09	3 35	4.30	0	3.30	
				1.0	3 72	.170
				2.0	3.92	.162
				4.0	4 13	.154
11/2	.12	2 35	3 75	0	2.10	
				1	2 80	.150
				2	3.10	.145
				4	3.45	145
10/13	1.0	2.35	4 5	0	2.35	
				10	3.20	.155
				20	3 60	.155
				40	4.00	.145

The results of the experiments are shown in Table II and Fig. 2. The values of C are again as constant as could be expected.

Cylinders.—The cylinders of gelatin were made by the repeated

dipping of a thread in liquid gelatin so that the gelatin on swelling did not increase in length but only in diameter. The average distance traversed by the water will be in this case $r/4$ and the total pore number Sn will equal $2\pi r_0 l$, where l is the length of the cylinder. In

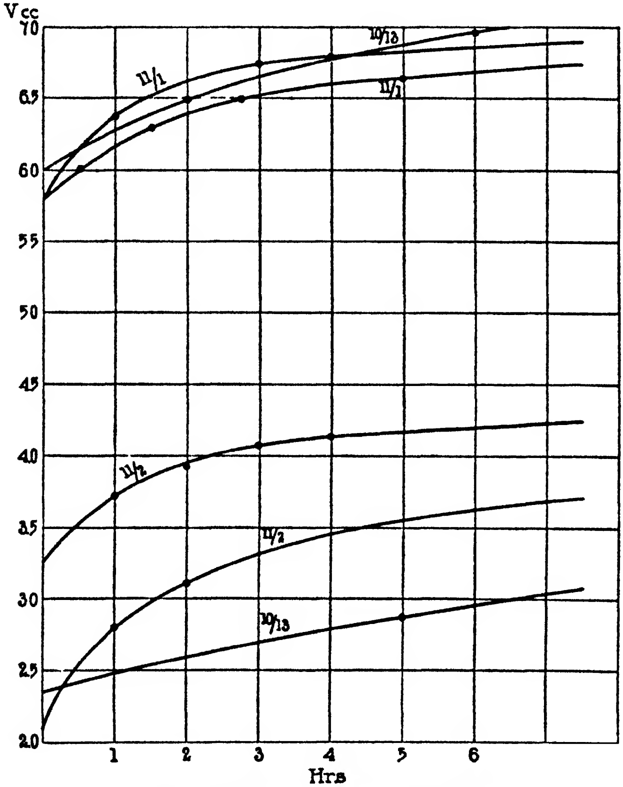

FIG. 3. Swelling of gelatin cylinders.

order to avoid fractional exponents it will be assumed again that $S = 2\pi r l$, and therefore $S/h = 4\pi l$. Substituting this value, equation (1) becomes

$$\frac{dV}{dt} = \frac{2 \times 1330 \times C \times 4\pi l(bV + V_f)(V_e - V)}{V_g \; VV_f V_e}.$$

Integrating and collecting constant terms the equation for the rate of swelling of cylinders is therefore:

$$C = \frac{gV_fV_e}{2 \times 1330 \times 4\pi lt}\left(\frac{V_e - V}{b} + \frac{2.3V_e^2}{bV_e + V_f}\log\frac{V_e - V_o}{V_e - V} + \frac{2.3V_f^2}{b^2(bV_e + V_f)}\log\frac{V_f + bV}{V_f + bV_o}\right)$$

The effect of swelling on the rate, owing to the simplifying assumptions used, is the same as for spheres but the effect of varying the initial size is different. The results of the experiment with cylinders are given in Table III and Fig. 3.

Direct Measurement of C.—According to the derivation of the equations, C should be the rate of flow of water in cc. per hour through a cylinder of gelatin having 1 cm. cross-sectional area, 1 cm. long, under a pressure of 1 mm. mercury. This value can be determined directly by measuring the flow of water through gelatin. Gelatin was allowed to solidify in glass tubes of 0.5 cm. diameter so as to form a plug 1 cm. long.[5] Water was then forced through these plugs under 20 cm. mercury pressure and the amount passing through measured in a pipette calibrated in 0.001 cc. The measurement was made at 5°C. Since when gelatin swells there is a loss of volume of the system as a whole, it is necessary to correct the observed rate for the change in volume when under no pressure. In the case of 14 per cent gelatin ($V_f = 6$), this correction is negligible; in the case of 23 per cent gelatin it is significant; and in the case of 30 per cent gelatin it is so large as to render the measurement uncertain. The values for the more dilute gelatin agree as well as could be expected with the value calculated from the rate of swelling measurement and show about the same effect of the original concentration. The plugs were then placed in water at

[5] It may be noted in this connection that the membrane cannot be held in place by a rigid support since in that case the pressure at first presses water out of the membrane. This continues until the osmotic pressure of the membrane itself is equal to the applied pressure. In other words, no matter what concentration of gelatin is used to make the membrane, water will be removed or taken up so that when a steady filtration rate is reached the concentration of gelatin in the membrane is that which will give an osmotic pressure equal to the applied pressure.

5° for 24 hours and the measurement repeated. The rate of flow was now so slow as to be impossible to measure with any accuracy, thus verifying directly the assumption made in the beginning that the permeability decreased with the increase in swelling. In the case of the direct measurement the gelatin was prevented from expanding by the glass tube so that it is not surprising that the effect of swelling is much more marked than in the case of blocks not enclosed in a solid wall. A summary of the values of C is shown in Table IV.

The results as a whole show that the equations fit the time rate

TABLE IV.

Summary Value of C.

$C \times 10^5$ = cc. per mm. Hg pressure, per hour, per sq. cm. surface per cm. thickness.

Concentration gelatin V_f	Cylinders	Plates	Spheres	Film on glass	Average	C by direct determination	
						Not swollen	Swollen
6 0	1 15	1 7	.9	(.13)			
	1.20	2.2	1.0	1.0			
	1.30			1.0			
				1.3	1.1	1.0	$<.10$
				.60			
				.50			
3.35	.16			.30	.2	.5	$<.10$
				.12			
2.35	.15						
	.15			.12	.13		

curves quite well. Owing to the number of constants and the uncertainty of the exact value for the equilibrium volume, this agreement might be regarded as accidental. What is much more significant, in the writer's opinion, is the fact that the equations lead to a value for the rate of diffusion of water through gelatin that has been checked by direct determination and also that they express correctly the effect on the rate of swelling of varying the initial size or shape of the block. It may be noted for instance that in the case of thin films the equation

predicts that the rate of swelling per gm. of dry gelatin decreases inversely as the square of the weight of dry gelatin, in the case of spheres it decreases approximately inversely to the 2/3 power of the weight of dry gelatin, while in the case of cylinders the rate is inversely proportional to the first power of the weight of dry gelatin. If the rate of swelling is expressed simply as the total amount of water taken in per unit of time, then the equation predicts that in the case of thin films the rate is inversely proportional to the size of the block, or if the surface is constant, to the thickness. This is the result obtained in all work on swelling.

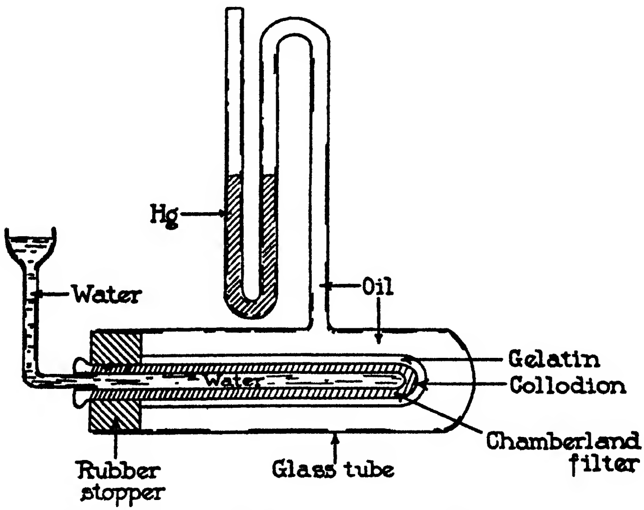

FIG. 4. Apparatus for measurement of swelling of gelatin on collodion-coated thimble.

Swelling under Experimental Conditions Which Avoid the Complicating Factors.

The preceding experiments show that the mechanism assumed for the swelling of gelatin predicts the results with considerable accuracy, but they are open to the objection that the formulas contain a number of constants and the agreement may therefore be accidental. If the mechanism is correct, however, it should be possible to predict con-

ditions under which the various secondary complications disappear or become negligible. These complications are due to the fact that the swelling pressure is opposed by the elasticity of the gelatin, a value which cannot be determined directly; and secondly to the fact that the resistance of the gelatin to the passage of the water is constantly increasing, due partly to the increase in the thickness of the layer of gelatin and partly to the decrease in the size of the pores. It was shown in the preceding paper[3] that at higher temperatures the elasticity is rapidly destroyed. It was also found that the resistance offered by collodion is very much greater than that offered by gelatin. If therefore a thin film of gelatin is coated on collodion so that the water has to pass through the collodion, the resistance offered by the gelatin will be a negligible part of the total resistance. If the experiment is carried out at 25° the elasticity of the gelatin will be partially destroyed and the term representing this force will also disappear. These conditions can be fulfilled by the apparatus shown in Fig. 4. The Chamberland filter was coated with collodion and then with gelatin. The gelatin was left at 5° for 24 hours in air and the apparatus set up as shown in a constant temperature bath at 25°C.

Under these conditions all the terms in equation (1) are constant except the pressure. The pressure will be equal to the swelling pressure minus the hydrostatic pressure. The curve for the swelling pressure of gelatin at 25° may be nearly superimposed on the curve at 5° by reducing the concentration of gelatin. The gelatin used had the same swelling pressure at 25° as 23 per cent gelatin at 5°, so that the same formula will apply for the pressure as was used for the experiments at 5° provided the concentration of gelatin be assumed to be 23 per cent. The swelling pressure is now opposed by the hydrostatic pressure just as in the osmotic pressure experiments, and the total pressure may therefore be written

$$P = OP - HP = \frac{1330 - 140V}{V} - M - Kg(V - V_o),$$

where M is the initial hydrostatic pressure and K is the mm. pressure per cc. increase in volume. Substituting this value of P, equation (1) becomes

$$\frac{dV}{dt} = \frac{CSnr^4}{h\eta g}\left[\frac{1330 - 140V}{V} - M - Kg(V - V_o)\right],$$

and bringing all the constant terms together and integrating:

$$C = \frac{2.3gV_e}{(1 + bV_e)\, 1330\, St}\left(V_e \log \frac{V_e - V_o}{V_e - V} - \frac{1}{b}\log\frac{1 + bV}{1 + bV_e}\right)$$

in which $b = KgV_e/1330$.

C in this case should be the cc. of water passing through 1 sq. cm. of the collodion-coated thimble per hour per mm. mercury pres-

TABLE V.

Swelling Gelatin on Collodion-Coated Thimble at 25°.

Experiment I. $g = 2.5$ $K = 220$ $V_e = 3.68$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.2	3.42	.45
.4	3.49	.51
.6	3.55	.57

Experiment II. $g = 2.0$ $K = 24$ $V_e = 5.3$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.4	3 78	1.05
.8	4.10	1.02
1.6	4.52	1.02
3.2	5 0	1.06
By direct determination		.1 2

sure. When the swelling experiment was concluded the gelatin was removed and this value of C was determined directly. The results of this experiment are given in Table V and Fig. 5. The difference in the values of C for the two experiments is presumably due to differences in the membranes. The value for the second experiment is quite close to the figure determined directly. At this temperature and under these conditions the final amount of swelling is not determined by the concentration of the gelatin but depends only on the hydrostatic

pressure. This confirms the assumption made above that at 25° the elasticity of the gelatin does not enter into the equation.

Secondary Swelling.

Under the conditions adhered to in these experiments an apparent maximum is rapidly reached. This value has been called the equilibrium volume. If measurements are carried on over a long period of time, however, it will be found that there is a slow steady increase in volume. This is shown in Fig. 6, in which the swelling has been plotted

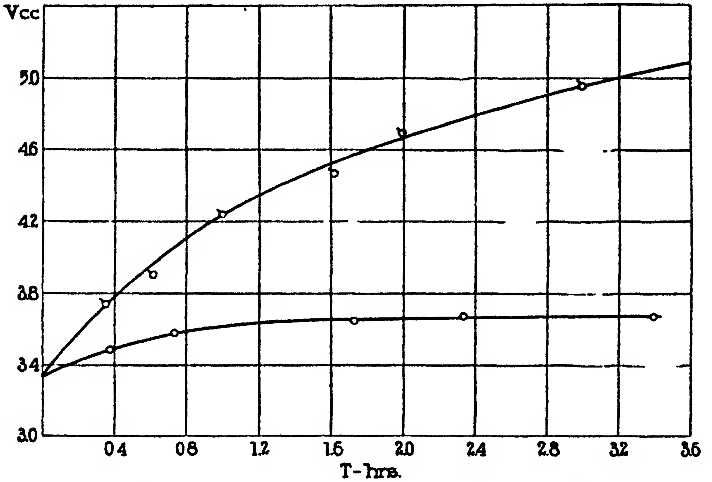

FIG. 5. Rate of swelling of gelatin on collodion-coated thimble.

against the square root of the time. This secondary swelling has been ascribed to the fatigue of the elastic force of the gelatin, and evidence was submitted in the preceding paper[3] to show that this was really the case. Fig. 6 shows that the rate of this secondary swelling is independent of the size of the block while the primary swelling is inversely proportional to the square of this quantity. This also bears out the idea that the secondary swelling is due to the fatigue of the gelatin, and hence is not regulated by the rate of diffusion of the water. This fatigue effect may be ascribed to a change in the value of V_f, which tends to approach the actual volume. It is possible to gain some idea

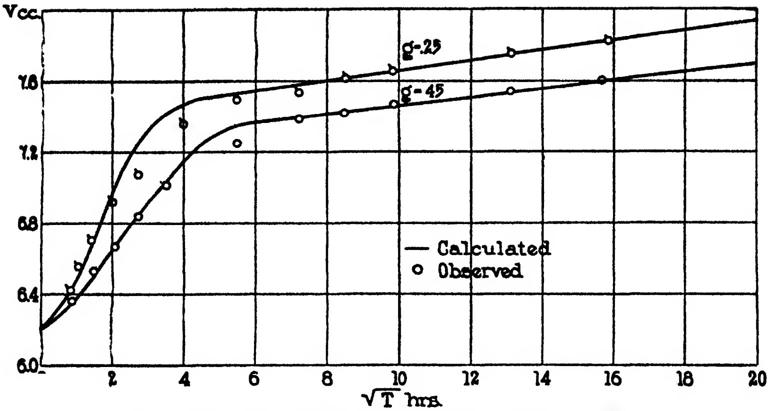

FIG. 6. Complete swelling curve of 13.5 per cent gelatin.

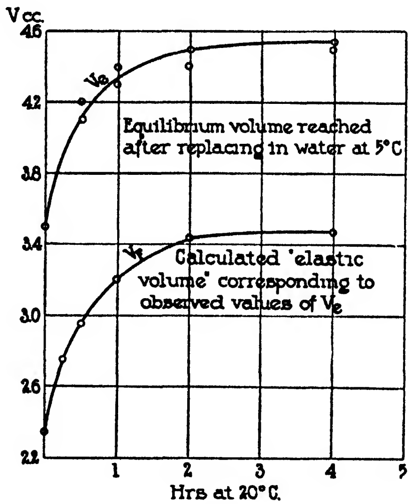

FIG. 7. Effect of time at 20° on subsequent swelling of swollen films of 30 per cent gelatin.

of the rate of change of this value by allowing swollen gelatin to remain varying lengths of time at 20° in air and then noting the swelling on returning the gelatin to water at 5°. The final volume reached in

water gives the value of V_e, and if the bulk modulus is constant the value of V_f at any time can be calculated. When these values of V_f are plotted against the time the block was kept in air, the second curve given in Fig. 7 is obtained. This curve is approximately logarithmic as might be expected and shows that the change of V_f with time is proportional to the difference between its value at time t and the equilibrium value V_{f_e}. Or

$$\frac{dV_f}{dt} = C(V_{f_e} - V_f),$$

which on integration becomes

$$C = \frac{1}{t} \log \frac{V_{f_e} - V_{f_0}}{V_{f_e} - V_f}.$$

Table VI shows that this equation holds at least as an approximation.

In this experiment the block was removed from so water that there was no change in the actual volume during the time V_f was changing,

TABLE VI.

Change of V_f with Time.

V_f–t		
	V_f	K
hrs		
0	2 35	
.25	2 74	.72
.50	2 98	.72
1.0	3 20	.62
4 0	3 47	

i.e. V_{f_e} was constant. In the actual experiments however, as soon as V_f changes V_{f_e} also increases. $V_{f_e} - V_f$ may therefore be considered as approximately constant, or

$$\frac{dV_f}{dt} = C', \text{ and } V_f = C't + \text{a constant}.$$

It was shown in the previous paper[3] that the equilibrium volume V_e was related to V_f by the equation $V_f = KV_e^2/1330 + V_e(K - 140)$.

The denominator of this equation may be considered constant for moderate changes in the value of V_e and the equation written

$$V_e^2 = C''V_f = C'''t; \tag{6}$$

or, since under these conditions V_e is the actual volume, $V = C\sqrt{t}$. This is the result shown in Fig. 6.

Complete Formula for Swelling.

Since the formula for the primary swelling has already been given the sum of this formula and formula (6) above will evidently represent the entire process. The equation for the primary swelling is too complicated to handle conveniently in this way, and it is necessary to omit some of the complicating factors. Since the primary swelling curve is basically logarithmic it is to be expected that it would fit the ordinary monomolecular formula provided the proper value of V_e is chosen. This of course deprives the formula of any theoretical meaning since V_e is actually determined by experiment, but may serve to give an expression for the first part of the primary swelling curve which can be used. This turns out to be the case, and it also happens that the value of V at which the secondary square root curve cuts the V axis may be used. The first part of the primary swelling may be represented therefore by the equation

$$Ct = \log \frac{V_e - V_o}{V_e - V'}, \tag{7}$$

where V' is the amount of swelling due to the primary process plus the original volume, or $V' = V_e - 10^{A-Ct}$; and the secondary swelling as

$$V'' - V_e = C\sqrt{t}, \tag{8}$$

where V_e in (7) is taken arbitrarily as the value of V_e in (8) when $t = 0$.

Since V' in equation (6) is the original volume plus the increase in volume due to the primary swelling, and $V'' - V_e$ is the increase in volume due to the secondary swelling, the total volume at any time will be the sum of these quantities or

$$V = C\sqrt{t} + V_e - 10^{A - C't}.$$

The solid lines in Fig. 6 were calculated by means of this formula and follow the general course of the actual experiment. In some of the experiments the fit was much better but there is naturally always a discrepancy near the end of the primary swelling curve.

It follows from the mechanism outlined above that if a large block is used, especially at a higher temperature, the primary swelling should be completely overshadowed by the secondary. That is, in a large block the outside layers will become fatigued and take in more water before the inside layers have swollen at all. The entire course of the

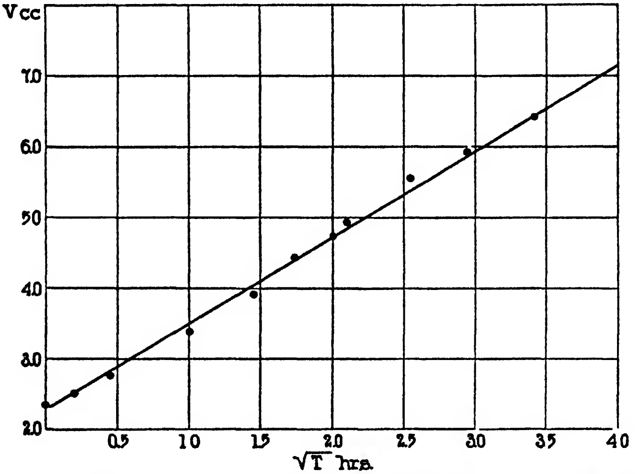

FIG. 8. Swelling of large block, 30 per cent gelatin at 27°. $g = 10$.

swelling should then be represented by the square root curve. The increase in weight of a block of 30 per cent gelatin containing about 35 cc., at 27°, is shown in Fig. 8. The process evidently follows the square root curve quite closely. According to this mechanism the gelatin should increase indefinitely or until it had taken up all the available water, and this is known to be the case.

Application to Other Types of Swelling.

If the theory of the kinetics of swelling outlined in this paper is correct it should apply in a general way to any system in which only

the solvent enters the material undergoing swelling, or in which the swelling is not affected by other substances present in solution. In the case of swelling due to a Donnan equilibrium, the pressure in the particle depends on the distribution of an electrolyte as well as on the entrance of the solvent. The theory would only apply to such systems provided conditions were such that the rate was determined by the passage of water into the solid. This condition is probably rarely if ever realized.

SUMMARY.

It has been assumed that gelatin consists of a network of an insoluble material enclosing a solution of a more soluble material.

The swelling of gelatin is therefore primarily an osmotic phenomena in that the water tends to diffuse in owing to the osmotic pressure of the soluble material. This osmotic pressure is opposed by the elasticity of the insoluble constituent, and equilibrium results when these two pressures are equal.

The rate of the entrance of water should then obey Poiseuille's law, provided the variable terms are expressed as functions of the volume. Equations have been derived in this way which agree quite well with the experimental curves and which predict the proper relation between the size and shape of the block and the rate of swelling. They lead to a value for the rate of flow of water through gelatin which has been checked by direct measurement.

The mechanism assumed predicts that at a higher temperature and under conditions such that the water has to pass through collodion before reaching the gelatin, the experiment should follow the same course as that of osmosis discussed previously. This was also found to be the case.

The slow secondary increase in swelling is ascribed to fatigue of the elastic properties of the gelatin. The rate of this secondary swelling should therefore be independent of the size of the block, in contrast to the rate of primary swelling which is inversely proportional to the size. It can further be shown that this secondary swelling should be proportional to the square root of the time, and also that with large blocks at higher temperatures the entire swelling should be of this secondary type. These predictions have also been found to be true.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1927, Vol. xi, No. 1, pp. 43–56.]

KINETICS OF THE SWELLING OF CELLS AND TISSUES.

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(Accepted for publication, June 8, 1927.)

Most cells or tissues when placed in distilled water increase in size owing to the entrance of the water into the cells and this swelling has frequently been compared to the swelling of simpler systems such as gelatin blocks. In preceding papers (1) formulæ have been derived which predict the course of the swelling of gelatin or the rate of entrance of water into a collodion membrane in a satisfactory way. If the swelling of plant or animal cells and tissues is really analogous to the swelling of gelatin or to the increase in weight of a membrane containing an osmotically active solution, it might be expected that the same formulæ could be used to predict the rate of swelling of cells or tissues. In the present paper data on the swelling of *Arbacia* eggs and on the swelling of slices of carrots or potatoes have been found to agree quite well with the formulæ referred to above.

Swelling of Arbacia Eggs.

The swelling of fertilized and unfertilized *Arbacia* eggs when placed in sea water diluted with tap water was studied by Lillie (2) who measured the increase in diameter of the eggs and computed the increase in volume. The results calculated to cc. are shown in Fig. 1 in which a smooth curve has been drawn through the experimental points. The figure shows that the fertilized eggs and the eggs on which fertilization membranes have been produced swell much more rapidly than the unfertilized eggs and soon reach a maximum. As Lillie points out, the increase in volume of the eggs should be proportional to the dilution of the sea water in which they are placed, if they are simple osmometers. Since the sea water was diluted 2½ times the eggs should increase in volume 2½ times. However they only double in size. Since the eggs

contain probably 20 to 30 per cent dry weight, and since it is the water content which should theoretically increase in proportion to the dilution of the sea water, this discrepancy would be partially corrected for if the calculation were made on a water basis rather than on the total volume. The expected increase in volume would also be less if the membrane were sufficiently elastic to oppose the entrance of the water. In the experiments of Lucke and McCutcheon (3), to be discussed later, it was found that the increase in size was nearly equal to that expected from the dilution of the sea water. It seems probable therefore that the force required to stretch the egg membrane is small and

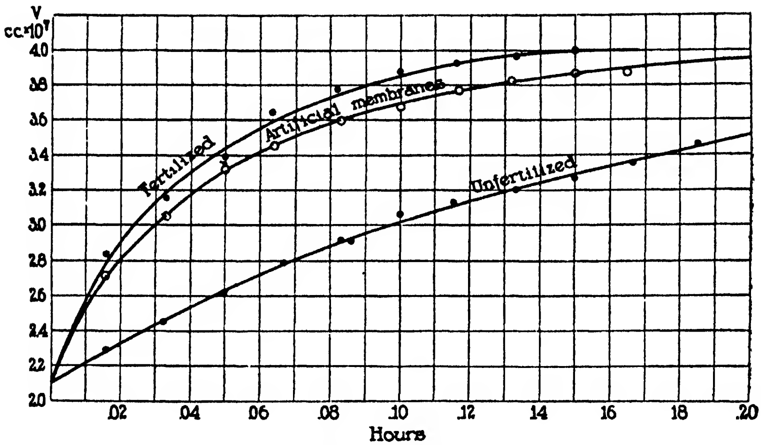

FIG. 1. Swelling of *Arbacia* eggs in 40 per cent sea water.

it has therefore been neglected in the following calculation. It has also been found by trial calculations that corrections for this elasticity or for the dry weight content of the egg fail to affect the results by more than the experimental error.

Lillie clearly recognized that the entrance of the water was a purely osmotic phenomenon, and that the rate was regulated by the permeability of the membrane and by the difference in the osmotic pressure of the inside and outside solutions. He assumed that all the other factors except the osmotic pressure difference were constant and therefore calculated the results on the basis of diffusion. He assumed, as

have other workers, that the rate of diffusion under these conditions should be proportional to the difference in pressure or,

$$\frac{dv}{dt} = K(P_e - P_m).$$

Lillie, in conformity with other workers, writes the integral of this equation as

$$K = \frac{1}{t}\, ln\, \frac{P_e - P_m}{P_t - P_m}$$

in which P_e is the initial pressure in the egg, P_m the pressure of the external solution and P_t the pressure at time t. It is then assumed that since the pressure is inversely proportional to the volume, the equation becomes

$$K = \frac{1}{t}\, ln\, \frac{v_{eq} - v_o}{v_{eq} - v_t}$$

where v_{eq} is the equilibrium volume. It appears to the writer, however, that in order to integrate the equation given above it is necessary to express P in terms of v *before* integrating. Substituting $\frac{P_o}{v}$ for P_e in the differential equation and adding the terms for the area and thickness of the membrane, the equation becomes

$$\frac{dv}{dt} = \frac{CS}{h}\left(\frac{P_o}{v} - P_s\right),$$

or

$$C = \frac{hv_e}{SP_o t}\left(v_o - v_t + 2.3\, v_e \log \frac{v_e - v_o}{v_e - v_t}\right), \qquad (1)$$

in which S is the surface of the membrane, P_o the "osmotic constant" of the solution inside the membrane, P_s the osmotic pressure of the external solution (assumed constant), v_e the volume at equilibrium, v_o the volume at the beginning of the experiment, h the thickness of the membrane and C the diffusion or "permeability" constant (1). In the units used by the writer C represents the cc. of water which will pass through 1 sq. cm. of membrane 1 cm. thick in 1 hour under 1 mm. Hg pressure. The equation has various forms depending on whether the

surface, thickness, and elasticity of the cell, and the osmotic pressure of the external solution, are considered constant as in this case. If the membrane is considered to be elastic or if the pressure of the outside solution changes, another term must be added. This equation has been found to agree with the rate of osmosis through collodion membranes (1).

Lillie's experiments have been calculated by this equation and the results are shown in Table I. The monomolecular constants are given for comparison. The values used for the volumes have been interpolated from the smooth curve since this procedure brings out better any drift of the constant which may be obscured by accidental variation in the case of the experimental points themselves. The table shows that the monomolecular constant decreases as Lillie stated in the case of the fertilized and artificial membrane eggs, while equation (1) gives constant values.

TABLE I.

Swelling of Arbacia Eggs in 40 Per Cent Sea Water after Lillie.

$v_o = 4.0 \times 10^{-7}$ cc. $v_o = 2.1 \times 10^{-7}$ cc. $S = 1.7 \times 10^{-5}$ sq. cm. $P_o = 4.6 \times 10^{-3}$ mm. Hg.

	Fertilized			Artificial membrane		
$v \times 10^7$	t	K_m	$\frac{C}{k} \times 10^6$	t	K_m	$\frac{C}{k} \times 10^6$
cc	*hrs.*			*hrs.*		
2 5	.0080	12.9	3 52	.0095	10 9	2 98
2 7	0130	12 7	3.62	0160	10 3	2.97
2 9	.0200	11.9	3 57	0240	10 3	2.97
3 1	0290	11 2	3 52	0350	9 3	2.91
3 5	0550	10 5	3 66	.070	8 4	2 92

The entrance of water into the eggs therefore follows the same course as the entrance of water into collodion membranes. In the case of the collodion membrane there is no increase in size of the membrane, and the surface and thickness are therefore constant. The eggs however increase in volume and the surface therefore also increases. It might be expected therefore that this factor would have to be considered, and that an equation derived on the assumption that

the surface was constant would not fit. Apparently, however, in the case of the fertilized eggs, this is not the case. The results indicate that the "diffusing surface" of the fertilized egg is not changed by the increase in volume or, if it is considered that the water enters through pores, that the number, radius and length of the pores is unchanged by the swelling.

The value of C/h is about 3×10^{-5}, which is about ten times smaller than the corresponding figure for collodion membranes (1). Since the egg membrane is probably less than one thousandth as thick as the collodion one, the permeability through the same thickness of membrane would be more than a thousand times as great for collodion as for the egg membrane. The results quantitatively confirm Lillie's statement that the egg membrane is relatively impermeable to water.

Unfertilized Eggs.

The course of the swelling of unfertilized eggs follows the monomolecular formula quite closely in Lillie's experiments. The swelling of unfertilized eggs has been studied in detail by Lucke and McCutcheon (3), and these workers also state that the swelling follows the monomolecular formula. Recalculation of their results shows a slight rise in the monomolecular constant in most cases (which however is probably hardly outside the experimental error), while the constant of equation (1) shows a definite increase. If the correction is made for the dry weight content of the egg, this increase in the monomolecular constant is slightly greater but even then is not marked. As stated above, this increase in the rate might be expected owing to the increase in surface of the egg. This effect can be taken into account in two ways, both of which however lead to the same equation. If the volume of the membrane is assumed to remain constant and the rate of diffusion is assumed proportional to the surface and inversely proportional to the thickness of the membrane, then, since the surface times the thickness equals the volume (which has been assumed constant), $S/h = 25v^{\frac{4}{3}}/m$, where m is the volume of the membrane. If, on the other hand, the water is supposed to flow through pores in the membrane, it may be assumed that the increase in surface during swelling is due to the increase in the size of the pores, and that the solid membrane surface remains practically constant. The surface of

the pores will be proportional to $5v^{2/3}$, and since according to Poiseuille's law the rate of flow through fine capillaries is proportional to the fourth power of the radius or to the square of the surface, the rate will be proportional again to $25v^{4/3}$.

The differential equation for the rate of swelling, then, neglecting the elasticity of the membrane and assuming that the osmotic pressure in the egg is inversely proportional to the volume of the egg, is

$$\frac{dv}{dt} = 25C_2\, v^{4/3}\left(\frac{P_o}{v} - \frac{P_o}{v_e}\right)$$

or, on integration,

$$C_2 = \frac{v_e^{2/3}}{25P_o t}\left[\left[1.15 \log \frac{v_e^{2/3} + v^{1/3} v_e^{1/3} + v^{2/3}}{(v^{1/3} - v_e^{1/3})^2} + \sqrt{3}\tan^{-1} - \frac{2v^{1/3} + v_e^{1/3}}{v_e^{1/3}\sqrt{3}}\right] - \left[1.15 \log \frac{v_o^{2/3} + v_o^{1/3} v_e^{1/3} + v_e^{2/3}}{(v_o^{1/3} - v_e^{1/3})^2} + \sqrt{3}\tan^{-1} - \frac{2v_o^{1/3} + v_e^{1/3}}{v_e^{1/3}\sqrt{3}}\right]\right], \qquad (2)$$

in which the second term represents the integration constant.

The results of some of Lucke and McCutcheon's experiments at different tempeiatures are given in Fig. 2, and the constants calculated by the monomolecular equation and by equation (2) in Table II. The monomolecular constants show a slight rise in most cases, while the constant of equation (2) shows in some cases a decrease.

It may be noted that if the water is supposed to flow through holes in the membrane then the constant C_2 contains the viscosity of water and should be corrected for the viscosity of water before comparing the values at different temperatures. It should also be corrected for the effect of temperature on the osmotic pressure, but since this is proportional to the absolute temperature this correction may be neglected for small changes in temperature. When the viscosity correction has been made the values of C given in the last row of Table II are obtained, and show a small and irregular temperature coefficient. The values of C in this equation cannot be compared directly with those obtained in equation (1), since they contain another constant representing either the volume of the membrane or the ratio of total surface to pore surface depending on which mechanism is assumed to be at work.

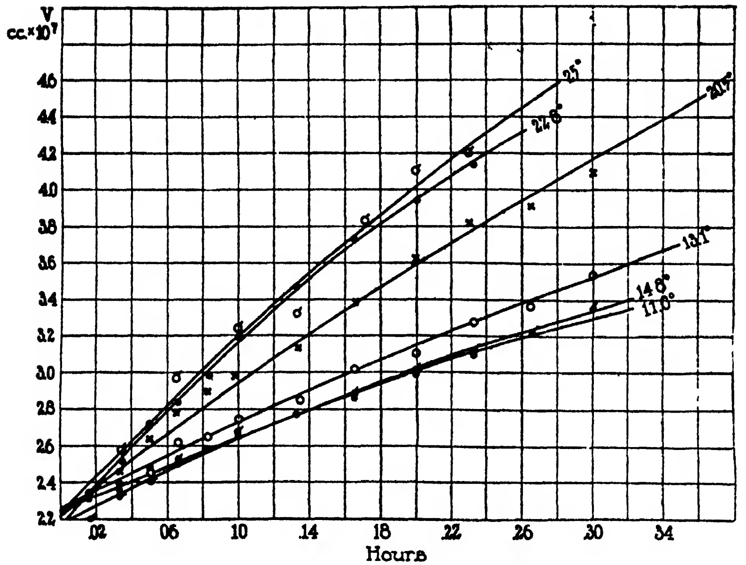

FIG. 2. Swelling of *Arbacia* eggs in 40 per cent sea water at different temperatures.

TABLE II.

Summary. Monomolecular and Osmotic Pressure Constants.

$v_o = 2.20 \times 10^{-7}$ cc. $v_e = 5.0 \times 10^{-7}$ cc. $P_e = 4.7 \times 10^{-3}$ mm. Hg.

	11°		13°		14 8°		20 5°		22 8°		25°	
$v \times 10^7$	K_m	$C_2 \times 10^3$	K_m	$C_2 \times 10^3$	K_m	$C_2 \times 10^3$	K_m	$C_2 \times 10^3$	K_m	$C_2 \times 10^3$	K_m	$C_2 \times 10^3$
cc.												
2 6	.82	1 50	.97	1 81	77	1 42	1.38	2 54	1.8	3 22	1.55	3.47
3 0	.77	1.41	93	1 69	78	1 41	1 42	2.62	1 86	3.35	1.69	3 45
3 4			96	1 67	.79	1 38	1.50	2 62	2.0	3 49	1.84	3.55
3.8								2 67		3 54		3 66
$C_2 \times 10^3 =$		1.45		1 72		1 40		2 60		3 40		3 53
$\eta \times 10^2 =$		1.30		1.22		1.14		1.00		.95		89
$C \times 10^5 =$		1 9		2 1		1 6		2.6		3 2		3 1

Effect of the Concentration of the Sea Waters.

Lucke and McCutcheon (3) also obtained some interesting results in connection with the effect of the concentration of sea water on the rate of swelling. They found that when the monomolecular constants for the process were compared, the constants decrease rapidly as the concentration of sea water in which the eggs were placed decreased. The permeability of the eggs to water was therefore apparently much less in dilute than in concentrated sea water. According to the mechanism of the process outlined above, the "permeability constant" C_2 is approximately proportional to the monomolecular constant times the volume at equilibrium, *i.e.*

$$C \sim K_m v_e.$$

TABLE III.

Rate of Swelling of Arbacia Eggs in Various Concentrations of Sea Water.

Per cent sea water	80	60	40	20
K.... . .	072	024	012	006
$v_e \times 10^9$ cc .	240	350	500	1050
Kv_e.....	17.3	8.4	6.0	6.3

When the values of K_m obtained by Lucke and McCutcheon are corrected in this way the values shown in Table III are obtained. They still show that the membrane is less permeable in 20, 40 and 60 per cent sea water than in 80 per cent, but the differences are not so marked as is the case with the monomolecular constant itself.

Swelling of Plant Tissue.

In the case of *Arbacia* eggs just considered the resistance to the entrance of the water is localized at the membrane, and in confirmation of this it was found that the kinetics of the reaction is analogous to that of water entering a collodion bag. In the case of material like gelatin, however, it is evident that the entire mass offers resistance to the passage of the water and that the resistance is not confined to the surface layers. It would be expected therefore that the swelling curves for

gelatin would be quite different from those for the passage of water into collodion sacs, and this was found to be the case. The swelling of slices of carrot or potato as studied by Stiles and Jørgensen (4) should presumably follow approximately the same course as that found for gelatin, since the structure of such tissues is very similar to that assumed for the gelatin.

Stiles and Jørgensen found that slices of potato or carrot neither lost nor gained weight when placed in M/4 sugar or in M/8 NaCl, lost weight in more concentrated solutions and gained weight in more dilute solutions. They assume that the tissue is permeable only to water under these conditions. The results of these experiments are shown

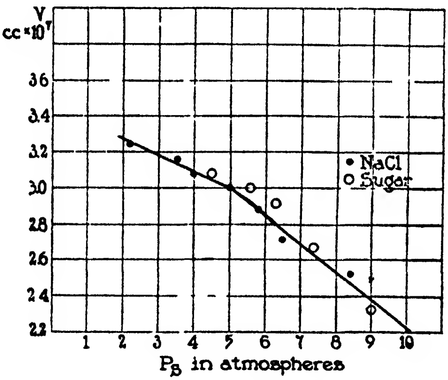

Fig. 3. Swelling of slices of potato in salt or sugar solutions.

in Fig. 3, in which the osmotic pressure of the NaCl in atmospheres as calculated from the freezing point depression (5) has been plotted against the volume of the potato slices expressed as gm. water per gm. dry weight. If it is assumed that the tissue is an elastic body, and that the water is drawn in by the difference in the osmotic pressure of the solutions inside and outside of the tissue, and further that this osmotic pressure is inversely proportional to the water content of the tissue, then the equilibrium condition will be defined (1) by the equation

$$\frac{P_e}{V_e} - P_s = \frac{K_e(V_e - V_f)}{V_f},$$

where P_e is the osmotic constant of the inside solution (*i.e.* $\frac{P_e}{V_e}$ equals the osmotic pressure of the salt solution which causes no change in volume), P_s is the osmotic pressure of the salt solution in which the tissue is immersed, V_e is the volume of water per gm. dry weight at equilibrium, V_f the volume when under no strain and K_e the bulk modulus. The values of K_e calculated from the data are shown in Table IV. Pressure which tends to remove water is called negative, and pressure required to add water, positive. The table shows that the value of K_e, which may be defined as the pressure in atmospheres required to change the volume by an amount equal to the volume of

TABLE IV.

Swelling of Potato in Various Concentrations of NaCl.

$V_f = 3.00 \quad P_e = 15.00$

$V = \frac{\text{gm } H_2O}{\text{gm. dry wt.}}$	NaCl Moles per 1000 gm. H_2O	Osmotic pressure NaCl Atmospheres	K_e Atmospheres
2 56	.20	8.4	17
2.72	.16	6 5	10
2 88	143	5 8	13
3 04	.125	5.0	
3 08	.10	4 0	35
3.16	.083	3 5	25
3 24	.050	2 2	33
3 68	.000	0	18

water originally present, is about 14 when the water is removed and about 30 when water is added. The values of K_e show quite large variations, but they are within the experimental error (except for the value in distilled water), since the equation is of such form that small errors in the experimental value are greatly exaggerated in calculating the value of K_e. In the case of distilled water the value suddenly decreases; that is, the tissue swells more than would be expected from the other results. This is the usual result with elastic substances when the elastic limit has been exceeded. The figures show that a very considerable force is required to either increase or decrease the size of the tissue and that as might be expected less force is required to contract than to expand it.

Kinetics of the Swelling Process.

The rate at which water enters the tissue was also determined by Stiles and Jørgensen. In the case of potato there seems to be some doubt as to whether a true equilibrium value is reached in distilled water, and the writer was unable to assign any very definite value to this equilibrium volume from the data published. The data for the swelling of slices of carrot, however, at various temperatures, show a definite maximum and these experiments have been used. Stiles and Jørgensen's results are shown graphically in Fig. 4, in which the increase in weight of slices of carrots in distilled water at 10°, 20° and

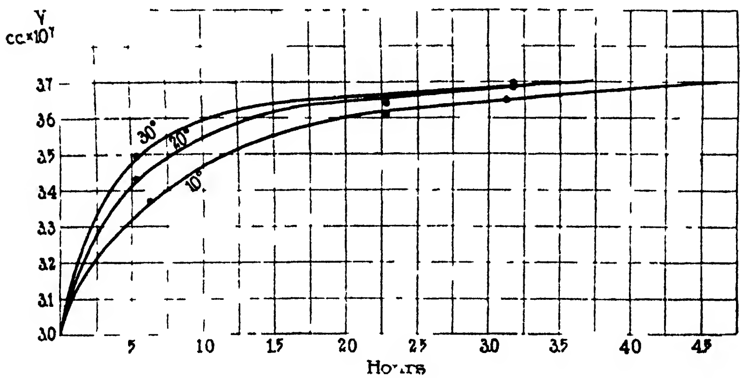

FIG. 4. Swelling of slices of carrot in distilled water at various temperatures.

30° expressed as gm. water per gm. dry weight has been plotted against the time in hours.

Stiles and Jørgensen considered that the water entered the cell owing to osmotic forces, and that this entrance was opposed by the elasticity of the tissue. They also point out that the process was probably analogous to the swelling of simpler systems such as gelatin blocks, so that the present treatment does little more than express their conclusions in mathematical form.

If the water is assumed to flow through small pores in the tissue (in this case possibly the spaces between the cells), in order to reach the

cells away from the surface then the rate of flow through the tissue as a whole will be expressed by Poiseuille's law

$$\frac{dv}{dt} = \frac{Cr^4\,SP}{\eta\,h},$$

in which v is the volume of water, r the radius of the pores, S the surface of the piece of tissue (assumed proportional to the number of pores), P the swelling pressure, η the viscosity of water at the temperature used, and h the distance through which the water has to diffuse C, the "permeability constant," will therefore be the cc. of water that will flow through 1 sq. cm. of tissue 1 cm. thick in 1 hour under 1 mm. Hg pressure. In the case of gelatin it was found that the rate of flow of water was less in swollen than in unswollen blocks, so that r^4 was assumed inversely proportional to V. In the case of slices S is constant and h is equal to $\frac{V}{2S}$, since the water enters from both sides and the average distance traversed by the water will be $\frac{1}{2}$ the thickness of the block. The swelling pressure will be equal to the difference between the osmotic pressure and the elastic force or

$$P = \frac{P_e}{V} - \frac{K_e(V_e - V_f)}{V_f}$$

In order to express the results as volume of water per gm. dry weight, let $v = Vg$ and hence $dv = gdV$ where v is the total volume of water, V is the volume of water per gm. dry weight and g is the weight of solid material. Substituting these values the differential equation becomes

$$\frac{dV}{dt} = \frac{2\,C\,S^2\,P_e\,(V_e - V)\,(bV + V_f)}{V^2\,V_f\,V_e\,g^2},$$

the integral of which is:

$$\frac{C}{\eta} = \frac{g^2\,V_e\,V_f}{2\,P_o\,S^2 t}\left[\frac{V_o^2 - V^2}{2\,b} + \frac{(b\,V_e - V_f)\,(V_o - V)}{b^2} + \frac{2.3\,V_e^3}{(b\,V_e + V_f)}\log\frac{V_e - V_o}{V_e - V}\right.$$
$$\left.\frac{2.3\,V_f^3}{b^3\,(b\,V_e + V_f)}\log\frac{V_f + b\,V}{V_f + b\,V_o}\right],$$

in which $b = \frac{K_e V_e}{P_e}$.

In this case V_e is the same as V_f since it is assumed that the tissue is under little or no strain before being placed in water. Table V shows the values of C calculated from the experiments by means of this formula. The last row gives the value corrected for viscosity. The table shows that the value is fairly constant for the different times, and also that there is little or no effect of temperature. This may be considered as indicating that the water does flow through fine pores and that the temperature coefficient is therefore that of the viscosity of water.

The foregoing results indicate that the formulæ derived for the swelling of gelatin may be used at least as a first approximation for

TABLE V.

Swelling of Slices of Carrot in Distilled Water at Various Temperatures.

$g = .45$ $P_o = 10\ 5 \times 10^3$ $V_o = 3\ 0$ $V_e = 3.70$ $K_e = 13 \times 10^3$ $S = 5.0$ sq cm.

V	10° $\frac{C}{\eta} \times 10^6$	20° $\frac{C}{\eta} \times 10^6$	30° $\frac{C}{\eta} \times 10^6$
3 32	9 55	14 9	19 1
3 47	9 37	13 3	18 8
3 60	8 70	13 2	16 8
Average $\frac{C}{\eta} \times 10^6$	9 2	13 5	18 2
$\eta \times 10^2$	1 31	1 00	800
$C \times 10^8$	12 0	13 5	14 5

the swelling of tissues and cells in certain cases. It may be pointed out that they cannot be used unless there is evidence that only water passes the membrane, that there is really a fairly constant bulk modulus, and unless the equation connecting the change in pressure with the increase in volume is known. In most of the experiments reported these conditions are not fulfilled. The swelling of *Xanthium* seeds for instance, studied by Shull (6), as well as results with other seeds, is difficult to interpret owing to the fact that the relation of the pressure to volume is not known and certainly cannot be assumed to be a simple inverse ratio as has been assumed for the present tissues which contain a very much larger amount of water originally. The writer has

found that by using an empirical exponential relation between the pressure and volume in *Xanthium*, the rate of swelling may be approximately calculated; but the method used really amounts to introducing two new arbitrary constants in the formula and deprives the results of their significance.

SUMMARY.

The rate of swelling of *Arbacia* eggs in dilute sea water, studied by Lillie and by Lucke and McCutcheon, may be expressed by the formulæ derived for the rate of increase in volume of a solution enclosed in a collodion sac.

The rate of swelling of slices of carrot in distilled water, measured by Stiles and Jørgensen, may be expressed by the equation derived previously for the swelling of similarly shaped blocks of gelatin.

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[Reprinted from The Journal of General Physiology, July 20, 1927, Vol. x, No. 6, pp. 927–947.]

ON THE NATURE OF THE DYE PENETRATING THE VACUOLE OF VALONIA FROM SOLUTIONS OF METHYLENE BLUE.

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(Accepted for publication, May 12, 1927.)

I.

INTRODUCTION.

Experimental results[1] favor the theory that a basic dye penetrates a living cell very rapidly in the form of free base (which predominates at a high pH value) but so slowly in the form of salt (which predominates at low pH values) that its penetration is comparatively negligible. If this theory were correct, we should not expect a dye like methylene blue, which is very strongly basic,[2] to enter a living cell, since at the range of pH values generally available for living cells this dye exists in the form of salts. Yet methylene blue is widely known as one of the most commonly used vital stains. What is the explanation for the discrepancy between the theory presented and the observed facts? Does this indicate that the theory is inadequate, or does it mean that the dye which penetrates is not methylene blue but a less basic lower homologue, such as azure B or trimethyl thionine,[3] which is found in methylene blue solutions

[1] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669 Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T B., *J. Biol. Chem.*, 1908, iv, 1. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350. Irwin, M., *J. Gen. Physiol.*, 1925–27, viii, 147; 1925–26, ix, 561; 1926–27, x, 75.

[2] For discussion of the apparent dissociation constant of methylene blue *cf.* Clark, W. M., and his collaborators (Clark, W. M., Cohen, B., and Gibbs, H. D., *Pub. Health Rep., U. S. P. H., No. 23*, 1925, 1131).

[3] The apparent dissociation constant of azure B has not been determined, but we have the following reason to assume that it is a weaker base than methylene blue. In general it is found that a substance whose amino groups are completely substituted with alkyl radicles, such as tetramethyl ammonium hydroxide,

especially at a higher pH value and which is capable of existing partly in form of free base? It has been stated by some investigators[4] that lower homologues are found in samples of methylene blue, and others[5] have found that in presence of air and with an alkaline reaction methylene blue in aqueous solution is partly converted to methylene

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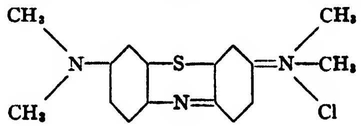
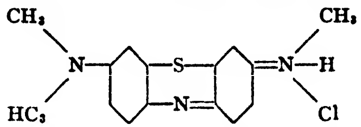
is a strong base, while a substance whose amino groups are only partially substituted by alkyl radicles, such as trimethyl ammonium hydroxide is a weaker base Since amino groups of methylene blue or tetramethyl thionine are completely substituted with alkyl radicles, while those of azure B or trimethyl thionine are only partially substituted by alkyl radicles, as shown below, it would seem reasonable to suppose that methylene blue behaves like a strong base while azure B behaves like a weaker base.

Methylene blue or tetramethyl thionine



Azure B or trimethyl thionine.



Difference between the chemical structure of the dye in form of free base and that of the dye in form of salt must be left undecided until further studies are made. It is uncertain as to whether such a difference between free base and salt as represented in the previous publication (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 76) is correct. It may very well be that the salt is represented by the structure given above and that the free base is represented by a structure in which the Cl is replaced by the OH, or it is an anhydro-base.

[4] Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. A delicate and reliable method for the detection of the presence of trimethyl thionine in methylene blue has been devised by Holmes and will appear in an early issue of *Stain Technology*. By means of this method it was found that the purest samples of methylene blue available for testing invariably contained small proportions of trimethyl thionine

[5] Bernthsen, A., *Ann. Chem*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxviii, 4.

azure, which was found[6] to enter chloroform and to appear red. Furthermore, Kehrmann[7] has stated that methylene azure, which is a mixture of trimethyl thionine (azure B) and asymmetrical dimethyl thionine (azure A), enters substances like ether, chloroform, and benzene in form of a base and not in form of a salt, while methylene blue is not soluble in ether.

In view of the fact that the dye from methylene blue solution does not enter[8] the living cells except when the pH value of the solution is high, we may have a good reason for suspecting that the dye capable of entering a living cell is not methylene blue (in the form of a salt), but a less basic homologue (in the form of a free base), just as in the case of absorption by a substance like ether (already discussed). In this case the theory first presented would prove adequate.

One way to test this question is to study by spectrophotometric analysis the nature of the dye inside and outside the living cell. Heretofore this has not been attempted. The writer therefore proposes to give in the present paper[9] a series of spectrophotometric analyses of the dye penetrating from solutions of methylene blue into the vacuole of the living cell of marine alga *Valonia macrophysa*.

II.

Penetration of Dye into Valonia from a Solution of Methylene Blue.

Details of technique will be omitted here since they have previously been given by the writer.[10] Mention may, however, be made of several points of importance. Medium sized cells were chosen to avoid errors caused either by injury or by contamination of the sap from the stained cell wall. If too large a cell was employed, it took so long for the dye to collect in the vacuole that injury often occurred before there was a sufficient quantity of dye in the sap for spectrophotometric analysis. If too small a cell was used, the dye derived from the stained cell wall when the cell was punctured by a capillary tube for the purpose of collecting the sap exceeded the concentration of dye in the vacuole, which had

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[6] *Cf.* Baudisch, O., and Unna, P. G., Foot-note 5.

[7] *Cf.* Kehrmann, Foot-note 5.

[8] Harvey, E. N., MacArthur, J. W., and Irwin, M., see Foot-note 1.

[9] A preliminary report of these analyses has been made by the writer (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425).

[10] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

penetrated from the external dye solution before the cell was removed from the solution and punctured.

It is desirable to possess a sound basis for judging the condition of cells during the experiment. It is not a difficult matter to determine an irreversible injury, but a reversible injury is almost impossible to detect. It was therefore necessary to have a more or less arbitrary basis for judging if the cells were injured. Cells during experiment were considered to be uninjured (1) if they continued to live after they were kept in the test solution for several hours beyond the time required for experiment, (2) if they were found to be living a day or so after they had been transferred from the test solution to normal sea water, (3) if the turgidity of the cell, as detected by touch, remained the same as that of control cells (as the cells become injured they lose their turgidity).

The pH values of the sea water employed were about pH 5.5, 9.5, and 10.9. The pH value of the Bermuda sea water in which the dye was dissolved was altered and determined in the following manner. To sea water, hydrochloric acid was added until the color of the test-tube containing the sea water and brom cresol purple matched that of the standard phosphate buffer solution at pH 6 containing the same concentration of the indicator and 0 6 M sodium chloride, which roughly corresponds to the halide concentration of Bermuda[11] sea water. To sea water sodium hydroxide was added until the color of the test-tube containing the sea water and cresol phthalein matched that of the test-tube containing standard borate buffer solution at pH 9.7 or at pH 11.2, containing the same concentration of the indicator and 0.6 M sodium chloride. Owing to the slight difference in the pH value of different samples of Bermuda sea water the volume of hydrochloric acid and sodium hydroxide added varied slightly, but to 100 cc. of sea water on an average was added 1.08 cc. of 0 2 M hydrochloric acid (for pH 5 5) or 0.8 cc. of 0.2 M sodium hydroxide (for pH 9 5) or 0 45 cc. of 0.5 M sodium hydroxide (for pH 10 9).

Since the addition of sodium chloride alters the pH values of the buffer solutions, the pH values of the standard phosphate and borate buffer solutions containing 0 6 M sodium chloride were determined by means of the hydrogen electrode, and they were found to be pH 5 5 (phosphate), pH 9.5 (borate), and pH 10.9 (borate). These pH values represent only approximate pH values of the given sea water, because the colorimetri determination is not accurate for the following reason. Since in the standard^ buffer solutions the halide content of the sea water is represented only by sodium chloride, and since some salts are known to change the color of the indicators more than the others, the pH value of the standard buffer solution containing 0 6 M sodium chloride and that of the sea water may not be exactly the same even though the color of the test-tube containing the one matches that of the test-tube containing the other.

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[11] Bermuda sea water contains about 0.58 M halides (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, vii, 637).

Furthermore, above pH 10.3, the magnesium in the sea water is precipitated, which in some cases interferes with colorimetric determination, and no indicators are very sensitive in this range, so that at pH 10.9 the error may not be small. But no effort was made to avoid such sources of error since only the relative pH values were desired for these experiments. These pH values therefore are sufficiently accurate to serve the purpose in the present case, though they are approximate values.

Methylene blue is partially salted out in sea water so that two kinds of solutions were used; (1) a dye solution in which all the precipitate was allowed to remain, (2) a dye solution from which the precipitate was in greater part removed by filtering. In both cases the concentration of the dye before and after the experiment was found to remain unchanged and approximately the same results were obtained.

The following samples of methylene blue were employed, which according to the writer's knowledge represent some of the purest available.

(1) $C_{16}H_{18}N_3SCl + 1H_2O$ sent by Dr Benda from Germany.

(2) $C_{16}H_{18}N_3SCl + 3H_2O$ sent by Dr. Benda from Germany.

(3) Sample F and Sample G, given by Dr. W. M. Clark and Dr. B Cohen of the Hygienic Laboratory, Washington, D. C.

(4) Bleu de methylen pour Bacteriologie, Microbiologie, Physiologie, Produit, given by Dr R. H. French of the Color Laboratory, United States Department of Agriculture, Washington, D. C.

(5) Merck's medicinal.

Owing to the difference in the solubility of different samples in sea water, different concentrations[12] varying from 0 01 to 0.04 per cent were employed.

In determining the concentration of dye in the sap the following method was used. Sap was collected by puncturing the cell (previously removed from the dye solution and wiped) by means of a sharp glass capillary tube, and by drawing up the sap from the vacuole. About 2 cc. of sap was then placed in a small test-tube and the color of this test-tube was matched with that of a test-tube of the same diameter containing a known concentration of methylene blue.

Merck's medicinal was used as the standard solution for all the samples employed because this was the only sample available in sufficient quantity to make up a series of standard solutions at different concentrations. When the concentration of dye in the sap was below 0.00004 per cent, the color appeared more greenish than the standard so that it was difficult to match the color Furthermore, above 0.0003 per cent the color of the test-tube containing the sap appeared more purplish than that of the standard so that it again became difficult to match.

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[12] Other concentrations were used as check experiments. In all cases, if any dye entered the vacuole of uninjured cells more entered from the external solution at pH 9 5 than at 5.5, provided the experimental errors described in the text are absent.

Experiments were carried out at 25° ± 0.5°C. in an incubator with air holes through which diffused light was allowed to enter.

When living cells of *Valonia* were placed in methylene blue dissolved in sea water at these two pH values it was found that at about pH 5.5 practically no dye penetrated the vacuole, while at about pH 9.5 more entered. For example, with Merck's medicinal methylene blue, after 1 hour, at pH 9.5, the concentration of dye in the sap was about 0.00006 per cent, while at pH 5.5 it was too dilute for determination. When other samples of methylene blue, already described, were used, it was found that with some samples more dye entered than from the Merck's medicinal, while from others less entered the vacuole. But in all cases the rate of penetration of the dye into the vacuole was higher with the external dye solution at pH 9.5 than at pH 5.5. But with the samples in which the penetration was extremely slow the amount of dye found in the vacuole was so small even after several hours of exposure that unless extreme care was taken there were possibilities of experimental error arising from (1) contamination of the sap from dye in the cell wall at the time of puncturing exceeding the actual penetration of dye into the vacuole before puncturing; (2) inability to match the color of the test-tubes accurately; (3) more rapid penetration of dye due to a slight and reversible injury which cannot be detected, and which may occur if experiments are extended for several hours or if the cells at the start are not in excellent condition (the dye enters more rapidly as the cells become injured).

These sources of error might in some cases cause the rate of penetration of dye to appear the same at pH 5.5 and at pH 9.5. Furthermore, since at pH 5.5 the cells become injured more rapidly than at pH 9.5, in some cases where the injury occurred to the extent of a very slight loss of turgidity the rate of penetration at pH 5.5 was found to be higher than that at pH 9.5.

Experiments have been made with cells which have been kept in stoppered glass bottles containing some sea water for several months, as well as with cells which have been kept in a pan of sea water for several weeks. In both cases it was found that so long as the cells were not injured, the dye entered more rapidly at pH 9.5 than at pH 5.5, though in the case of the cells which have been kept in the laboratory for several months, as described above (cells appeared

less green than more recently collected cells), the dye entered more rapidly than in the case of the cells which have been kept only for a few weeks.

When the sap collected from the uninjured cells which had been exposed to the dye solution was oxidized by shaking and exposing to air with an alkaline reaction, no increase in coloration took place so that we may conclude that there was no dye in reduced form present in the sap.

When the pH value of the sap was determined after the living cells of *Valonia* had been exposed to sea water at pH 5.5, 9.5, and 10.9 for 4 hours, no change in the pH value of the sap occurred if the cells were not injured.

III.

Spectrophotometric Analysis.

The nature of the dye in the external solution and in the vacuolar sap of uninjured and injured cells was tested by means of spectrophotometric determinations. The measurements were made at the Color Laboratory in Washington, D. C., by W. C. Holmes, of whose collaboration the writer desires to express her appreciation.

The instrument employed was a Hilger wave-length spectrometer, equipped with a Nutting photometer. Either 1 or 2 cm. layers of solution were examined, depending on the concentration of dye in the solutions and the quantities of solution available. The measurements were carried out over the spectral range between 540 and 690 mμ. The concentration of dye was adjusted, insofar as was possible, to afford maximum visual sensitivity at and near the absorption maximum of the dye in dilute aqueous solution. All recorded values in this restricted region are averages of a considerable number of measurements.

A brief statement of spectroscopic criteria is advisable at this point. The absorption maximum of methylene blue in dilute aqueous solution is approximately 665 mμ. The corresponding maximum of trimethyl thionine is approximately 650 mμ. Although the average visual sensitivity in this region of the spectrum is relatively inferior it is readily possible to locate absorption maxima (with favorable dye concentrations) within a possible variation of about $\pm$1mμ.

The determination of the approximate absorption maximum, accordingly, differentiates the two dyes with absolute certainty. It affords, moreover, a reliable, if somewhat rough, criterion of the relative proportions of the dyes in question when both are present. Owing to the relatively limited spectral interval between their bands the band of a mixture of the dyes does not inhibit the individual maxima of its two component bands, but, rather, a single composite maximum of which the location varies with dye proportions. The absorption maximum of a mixture containing 66 per cent of methylene blue and 33 per cent of trimethyl thionine, for example, falls at approximately 660 mμ, while that of a mixture of 33 per cent of methylene blue and 66 per cent of trimethyl thionine falls at approximately 655 mμ.

It may be noted that the employment of suitable spectrophotometric ratios would afford a more exact definition of relative dye proportions. The basic data requisite for this procedure, however, were not available when the present investigation was begun and it was felt that the mere determination of the approximate absorption maxima of solutions would afford ample evidence of their character for present purposes.

Both methylene blue and trimethyl thionine exhibit secondary absorption in the general spectral region near 600 mμ. Both dyes are held to exist in aqueous solutions in a state of tautomerism between two dye forms.[13] In the present investigation considerable variations were noted in the apparent tautomeric equilibria between dye forms. These arise primarily from variations in dye concentration and are also influenced by other factors. A discussion of these phenomena is unnecessary in this paper. It is sufficient to note that their occurrence does not modify in any appreciable degree the relative absorption of the dyes at different wave-lengths within the critical spectral region between 650 and 665 mμ, or invalidate conclusions derived from variations in absorption within that region.

When such analyses were made some very interesting facts were obtained, as follows:

I. A sample of methylene blue,[14] dissolved in (1) Bermuda sea

[13] Holmes, W. C., *Ind. and Eng. Chem.*, 1924, xvi, 35; *Stain Technol.*, 1926, i, 17.

[14] Several samples were employed (see Section II in text).

water, (2) sap of *Valonia macrophysa,* and (3) artificial sap,[15] gave absorption maxima[16] characteristic of methylene blue, about 665 mμ (see Table I and Fig. 1, Curves *A*, *B*, and *C*).

II. The dye allowed to diffuse out of the cell wall (which had been previously stained by placing the living cells for a few minutes in sea water containing methylene blue), into artificial sap of *Valonia* gave

TABLE I.

Solutions	Primary absorption maximum
	mμ
Methylene blue dissolved in sea water	665
Methylene blue dissolved in the sap of *Valonia*	665
Methylene blue dissolved in artificial sap of *Valonia*	665
Dye which has diffused out of the cell wall into artificial sap, after the cell wall of living cells of *Valonia* has been stained in methylene blue (dissolved in sea water).	665
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5.	663
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 5.5	665
Trimethyl thionine dissolved in sap of *Valonia*	650
Dye found in the vacuole of uninjured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 9.5· This was freed from chloroform by absorbing it in distilled water	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 5.5: This was freed from chloroform by absorbing it in distilled water	655
Methylene blue dissolved in distilled water.	665
Dye absorbed by chloroform from methylene blue dissolved in M/150 buffer mixtures at pH 5.5 or at pH 9.5· This was freed from chloroform by absorbing it in distilled water	650

the absorption maximum of 665 mμ characteristic of methylene blue (Table I and Fig. 1, Curve *D*).

[15] The pH value of the sap is bout 5.8. The sap contains about 0.6 M halides (*cf.* Osterhout, W. J. V., and Dorcas, M. J., Foot-note 11).

[16] The absorption curve thus obtained resembles that of a higher concentration of methylene blue dissolved in distilled water. This may be due to the effect of salt on the dye, as suggested by Dr. W. C. Holmes.

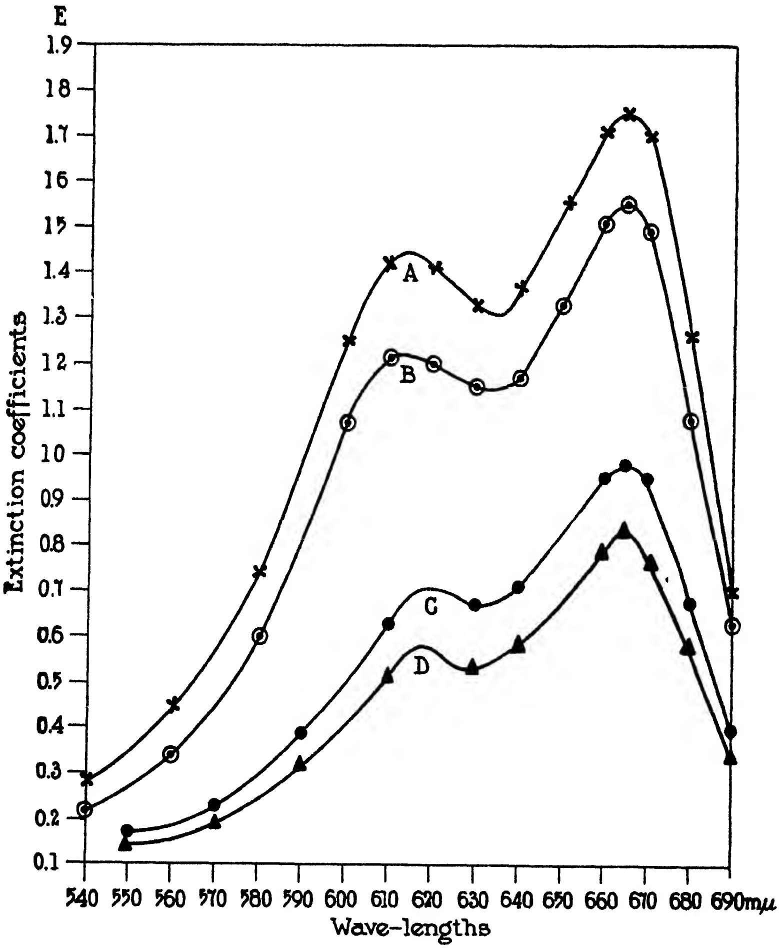

FIG. 1. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curve *A* represents the methylene blue dissolved in sea water, Curve *B* in the sap of *Valonia*, Curve *C* in the artificial sap of *Valonia*, Curve *D* the dye that has diffused from the cell wall into artificial sap after the cell wall of living cells of *Valonia* has been stained in methylene blue dissolved in sea water.

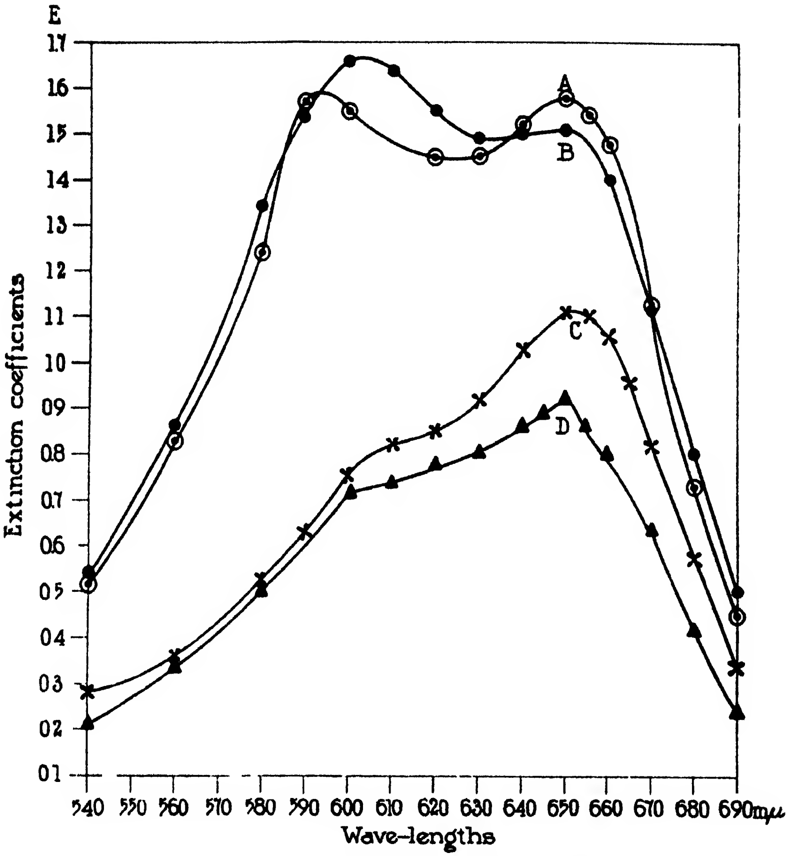

FIG. 2. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves A and C (symbols ⊙ and ×) represent the dye which was found in the vacuolar sap of uninjured cells of *Valonia* after the cells have been stained for 1 hour in one sample of methylene blue dissolved in sea water at pH 9.5 (Curve A) and in another purer sample at pH 10.9 (Curve C) Curves B and D represent the sample of trimethyl thionine or azure B dissolved in sap of *Valonia* at two dilutions, corresponding approximately to two dilutions represented by Curves A and C respectively. Curve B corresponds with Curve A and Curve C with Curve D.

III. Two concentrations of trimethyl thionine or azure B (sent by W. C. Holmes) dissolved in the sap of *Valonia* (Table I, Fig. 2, Curves *B* and *D*) gave absorption maxima of 650 mμ. This sample is obtained by the oxidation of methylene blue. Holmes suggests that though it is shown to be a fairly pure product it is possible that it contains small proportions of both methylene blue and asymmetrical dimethyl thionine.

IV. The dye in the sap collected from the vacuole of uninjured cells after an exposure of 1 hour to sea water saturated with two samples of methylene blue[17] (1) 0.04 per cent at pH 9.5 (Curve *A*, Fig. 2), and (2) 0.01 per cent at pH 10.9 (Curve *C*, Fig. 2). In Curve *A* the absorption maximum is 650 mμ which shows that the dye is chiefly trimethyl thionine and it gives no visible evidence of the presence of methylene blue. The absorption maximum of Curve *C* is about 652 mμ which shows that there is a trace of methylene blue, though the dye is chiefly trimethyl thionine. The presence of a trace of methylene blue in all probability is due to the contamination of the sap from the stained cell wall at the time the cell was punctured to collect the sap. Such a contamination plays an important part whenever the concentration of the dye in the sap is relatively small.

V. The dye collected from the vacuole of injured cells (slightly soft) after 12 hours' exposure to sea water saturated with methylene blue, either at (1) pH 5.5, or (2) at pH 9.5 gave an absorption maximum of 665 mμ (methylene blue) for (1) and 663 mμ (methylene blue and a little azure B) for (2) (Table I, and Fig. 3, Curves *A* and *B*). Since in both cases the dye in the sap collected from the vacuole was diluted with the sap collected from the vacuole of unexposed living cells, the heights of the curves which vary with dilution (the higher curve corresponding to the higher concentration) given in Fig. 3 do not show true relative concentrations of the dye found in the vacuole.

The azure B found by spectrophotometric analysis in the sap collected from the vacuole of uninjured cells of *Valonia* is not due to the transformation of methylene blue into azure B after methylene blue

[17] Samples employed are specified in the text in Section II. Owing to the fact that the purpose of these experiments is not to determine the purity of these samples, the sample used for each result is not specified.

has penetrated from the external dye solution into the vacuole because not enough conversion takes place during 1 to 3 hours in the methylene blue dissolved in the sap of *Valonia* to be detected by this method.

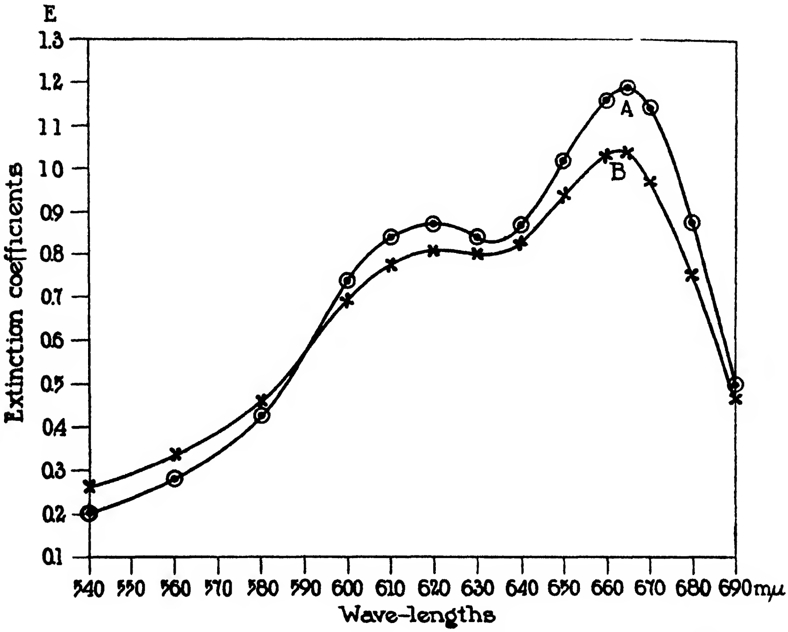

FIG 3. Extinction coefficients are plotted as the ordinates and the wavelengths as the abscissæ. Curves representing the dye in the sap collected from the vacuole of injured cells of *Valonia* and diluted with sap after the cells have been stained in the methylene blue dissolved in sea water, Curve *A* at pH 5 5 and Curve *B* at pH 9.5.

IV.

Absorption of Dye by Chloroform from Methylene Blue Solution.

In view of the fact that a similarity was found between *Valonia* and chloroform in their behavior toward other basic[18] dyes, in that

[18] When living cells of *Valonia* were placed in different basic dyes, Lauth's violet, neutral red, and brilliant cresyl blue, it was found that the higher the pH values of the dye (*viz.* between pH 5 and pH 8), the more rapidly the dye

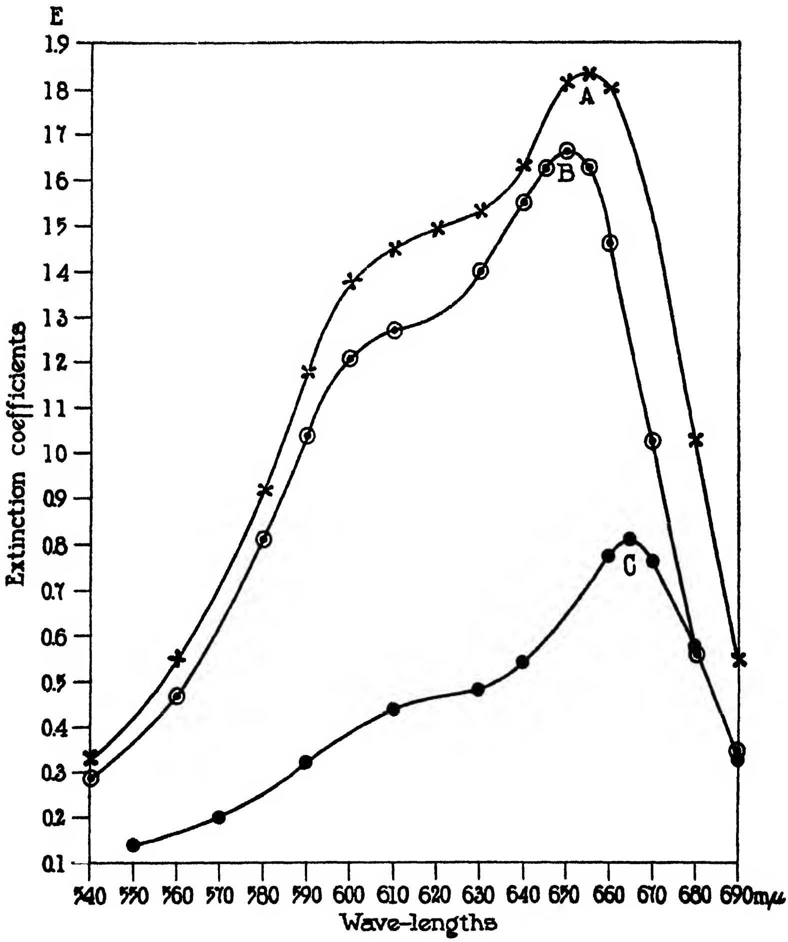

FIG. 4. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *B* represent the dye which was absorbed by chloroform from methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9. In both cases the dye was freed from chloroform by its subsequent absorption in distilled water. Curve *C* represents methylene blue dissolved in distilled water.

both take up the dye in the form of free base much more readily than in the form of salt, it seemed possible that chloroform, like *Valonia*, takes up chiefly trimethyl thionine or azure B from a solution of methylene blue, which may be determined by means of spectrophotometric analysis and by the determination of the partition coefficient of the dye between chloroform and water.

Heretofore no spectrophotometric analysis of the dye absorbed by chloroform from methylene blue has been made. This was accordingly done in the case of the dye absorbed by chloroform from a solution of methylene blue (made up in sea water). From chloroform thus stained, dye was freed by subsequent absorption in distilled water. When the sea water was at pH 9.5, the dye absorbed by chloroform gave the absorption maximum 650 mμ, characteristic of azure B (Table I, and Fig. 4, Curve *B*), and at pH 5.5 an absorption maximum of 655 mμ (which showed that there was a small amount of methylene blue in addition to azure B, Table I, and Fig. 4, Curve *A*). The methylene blue dissolved in distilled water gave an absorption maximum of 665 mμ (Table I, Fig. 4, Curve *C*).

The dye absorbed by chloroform from aqueous methylene blue solution (made up with M/150 buffer mixtures) was found to be azure B, both at pH 5.5 and at pH 9.5 (Table I).

These analyses show that in the presence of sea water at pH 5.5 azure B and a small amount of methylene blue are absorbed by chloro-

entered the vacuole. Relative rate of penetration differed with various basic dyes. Such differences in the rates corresponded roughly with the differences in the degree of absorption of these dyes by chloroform at different pH values of the sea water, and in the basicity of the dyes. When the amount of brilliant cresyl blue absorbed by chloroform or by *Valonia* is plotted against the external pH values, an S-shaped curve is obtained in both cases.

In view of the fact that in presence of sea water a basic dye in form of salt, as well as in form of free base, enters the chloroform, it is difficult to obtain with any accuracy the distribution coefficient of the dye only in form of free base between chloroform and sea water. This complication, however, was absent in the case of the basic dyes dissolved in M/150 buffer solutions, so that it was possible to make a comparison on a quantitative basis between the absorption of dye in form of free base by the vacuole of *Nitella* and by chloroform (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561).

form, while from sea water at pH 9.5 and from dilute buffer solutions at both pH values chiefly azure B is absorbed.

As to the experiments on the distribution of the dye between methylene blue dissolved in sea water and chloroform the following results were obtained.

When 3 cc. of chloroform were shaken up with 10 cc. of 0.002 per cent methylene blue dissolved in sea water at pH 9.5 and pH 5.5, it was found that the apparent partition coefficient of the dye between chloroform and sea water at pH 9.5 was about 2 (*i.e.* more concentrated in chloroform), while at pH 5.5 it was about 1.3. These might be called the apparent partition coefficients, since they merely represent the ratio of the concentration of the dye in the chloroform to that of the dye in sea water, without taking into account whether the dye is azure B, methylene blue, or a mixture of both.

The color of the dye in the chloroform when absorbed from methylene blue in sea water at pH 9.5 is reddish purple and from methylene blue in sea water at pH 5.5 is blue. Since the dry salt of methylene blue and that of azure B dissolved in chloroform is blue, the dye in the chloroform absorbed from methylene blue in sea water at pH 5.5 may possibly represent a mixture of methylene blue and azure B, in form of salt, while the dye in chloroform absorbed from methylene blue in sea water at pH 9.5 is azure B chiefly in form of free base. These experiments are insufficient to show whether or not these dyes in form of salt enter chloroform as undissociated molecules which may possibly be formed to a certain extent in presence of so high a concentration of salt such as sodium chloride in the sea water.

Just as in the case of the vacuole of uninjured cells of *Valonia* the dye which is taken up by chloroform from methylene blue in sea water at pH 9.5 is chiefly azure B in form of free base and not methylene blue.

Whether methylene blue is capable of penetrating the vacuole from methylene blue in sea water at pH 5.5 cannot be determined, since the dye does not penetrate in sufficient quantity for spectrophotometric analysis. Since the concentration of undissociated molecules of methylene blue in form of salt possibly present in sea water is not determined, this result neither proves nor disproves the theory that the undissociated molecules enter the vacuole of uninjured cells more rapidly than the ions.

It is not certain as to whether methylene blue enters the chloroform in constant amount at all pH values or only at lower pH values. Further experiments are necessary to determine this point.

V.

Penetration of Azure B into the Vacuole of Valonia and into Chloroform.

If our supposition is correct that azure B is a weaker base than methylene blue, and capable of existing in form of free base at higher pH values, then, according to the theory presented in Section I, a pure sample of azure B should penetrate into the vacuole of *Valonia* and into chloroform more when the pH value of the sea water is higher.

Living cells of *Valonia* were therefore placed for ½ hour in 0.04 per cent azure B (1) made by Holmes, (2) extracted by chloroform from methylene blue solution (made up with borate buffer at pH 9.5). With both samples approximately the same results were obtained, in that at pH 9.5 the rate of penetration was much higher (about 0.001 per cent dye in sap) than at pH 5.5 (dye in sap was too dilute for accurate determination).

When 3 cc. of chloroform were shaken up with 10 cc. of the azure B dissolved in sea water at pH 9.5 and at pH 5.5, the partition coefficient of the azure B (made by Holmes) between chloroform and sea water at pH 9.5 was 14.9 and at pH 5.5 was 1.8 (*i.e.* the dye was more soluble in chloroform than in sea water).

The color of the dye in chloroform when sea water was at pH 9.5 was reddish purple, while it was blue at pH 5.5.

Since the dry salt of azure B dissolved in chloroform appears blue, the azure B taken up by chloroform from sea water at pH 5.5 may be in form of salt, and at pH 9.5 in form of free base (which is reddish purple).

VI.

DISCUSSION.

From these results we may conclude that the vacuole of uninjured cells of *Valonia macrophysa* takes up chiefly trimethyl thionine (azure B) from the solution of methylene blue which contains so little azure B (as impurity) that it cannot be detected by the spectro-

photometer. As soon as cells are injured methylene blue enters. The writer's results and conclusion are contrary to those obtained by M. M. Brooks,[19] who states that the vacuole of uninjured cells of *Valonia macrophysa* takes up dye from methylene blue solution with the same speed at all pH values (from pH 5 to 9), from which she concludes (without analysis of the dye in the vacuole) that methylene blue (in form of salt) enters the vacuole of uninjured cells.

The writer's experiments show that the penetration of dye into the vacuole of uninjured cells from a solution of methylene blue does not discredit the theory that the basic dye enters the vacuole chiefly in the form of free base, since the dye which penetrates is found to be chiefly a lower homologue of methylene blue, azure B, which is less basic and capable of existing in part in the form of free base at higher pH values. Azure B behaves like all other basic dyes in that its relative rate of penetration depends on the amount of dye in form of free base present, which corresponds with the pH value of the external solution (the higher the pH value the more dye is in form of free base and the more rapid is the rate of penetration). That this difference in the rate of penetration at varying pH values is not due primarily to the effect of different pH values on the protoplasm is shown by the fact that the relative rates of penetration at a given series of pH values differ with different basic dyes.

The writer's previous statement that the vacuole of living cells, such as that of *Valonia*, behaves very much like chloroform toward basic dyes, in that they both take up the dye in the form of free base, is still further supported by the fact that they both take up primarily azure B from methylene blue solution at higher pH value.

Undoubtedly the penetration of a basic dye depends chiefly on two factors under such experimental conditions, (1) on the apparent dissociation constant of the dye, (2) on the partition coefficient of the dye between the vacuolar sap and the external solution, and in some cases on that of the dye between the vacuolar sap and the protoplasm. In case there is a combination of dye with some constituent of the sap, this factor must be brought into consideration. With chloroform also penetration depends on the dissociation constant and the partition coefficient.

[19] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

There is a similarity between chloroform and the vacuole of uninjured cells of *Valonia* in that they are both capable of taking up azure B and some other basic dyes in form of free base, but in certain cases chloroform and *Valonia* are found not to behave alike. For example, some acid dyes are slightly soluble in chloroform but they do not penetrate the vacuole of uninjured cells of *Valonia*. However such an analogy is not complete since the ability of the dye to collect in the vacuole may not only depend on the ability of the protoplasmic layer (between protoplasm and external solution, or between protoplasm and the vacuole) to absorb the dye but also on its power to give up the dye. Experiments are being done with this consideration in view.

The fact that azure B instead of methylene blue is found in the vacuole is not proof that methylene blue does not enter the protoplasm. It might enter the protoplasm though it does not penetrate into the vacuole. One way to arrive at a definite conclusion is to determine the nature of the dye inside the protoplasm, after the dye has been allowed to penetrate the cell in uninjured condition; but with the protoplasmic layer of *Valonia* this cannot be accomplished since it cannot be removed for examination without contamination or injury. Furthermore there is no way of determining whether or not methylene blue enters protoplasm and is converted to azure B or trimethyl thionine.

These experiments show the danger of drawing conclusions as to permeability or as to oxidation-reduction potentials from the experiments on the penetration of dye from a solution of methylene blue into living cells, unless we know the nature of the dye both in the external solution and inside the cells.

These experiments were repeated with *Nitella* and gave approximately the same results.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

SUMMARY.

When uninjured cells of *Valonia* are placed in methylene blue dissolved in sea water it is found, after 1 to 3 hours, that at pH 5.5

practically no dye penetrates, while at pH 9.5 more enters the vacuole. As the cells become injured more dye enters at pH 5.5, as well as at pH 9.5.

No dye in reduced form is found in the sap of uninjured cells exposed from 1 to 3 hours to methylene blue in sea water at both pH values

When uninjured cells are placed in azure B solution, the rate of penetration of dye into the vacuole is found to increase with the rise in the pH value of the external dye solution.

The partition coefficient of the dye between chloroform and sea water is higher at pH 9.5 than at pH 5.5 with both methylene blue and azure B. The color of the dye in chloroform absorbed from methylene blue or from azure B in sea water at pH 5 5 is blue, while it is reddish purple when absorbed from methylene blue and azure B at pH 9 5. Dry salt of methylene blue and azure B dissolved in chloroform appears blue

It is shown that chiefly azure B in form of free base is absorbed by chloroform from methylene blue or azure B dissolved in sea water at pH 9 5, but possibly a mixture of methylene blue and azure B in form of salt is absorbed from methylene blue at pH 5 5, and azure B in form of salt is absorbed from azure B in sea water at pH 5 5.

Spectrophotometric analysis of the dye shows the following facts.

1. The dye which is absorbed by the cell wall from methylene blue solution is found to be chiefly methylene blue

2 The dye which has penetrated from methylene blue solution into the vacuole of uninjured cells is found to be azure B or trimethyl thionine, a small amount of which may be present in a solution of methylene blue especially at a high pH value.

3 The dye which has penetrated from methylene blue solution into the vacuole of injured cells is either methylene blue or a mixture of methylene blue and azure B.

4. The dye which is absorbed by chloroform from methylene blue dissolved in sea water is also found to be azure B, when the pH value of the sea water is at 9.5, but it consists of azure B and to a less extent of methylene blue when the pH value is at 5.5.

5. Methylene blue employed for these experiments, when dissolved

in sea water, in sap of *Valonia*, or in artificial sap, gives absorption maxima characteristic of methylene blue.

Azure B found in the sap collected from the vacuole cannot be due to the transformation of methylene blue into this dye after methylene blue has penetrated into the vacuole from the external solution because no such transformation detectable by this method is found to take place within 3 hours after dissolving methylene blue in the sap of *Valonia*.

These experiments indicate that the penetration of dye into the vacuole from methylene blue solution represents a diffusion of azure B in the form of free base. This result agrees with the theory that a basic dye penetrates the vacuole of living cells chiefly in the form of free base and only very slightly in the form of salt. But as soon as the cells are injured the methylene blue (in form of salt) enters the vacuole.

It is suggested that these experiments do not show that methylene blue does not enter the protoplasm, but they point out the danger of basing any theoretical conclusion as to permeability on oxidation-reduction potential of living cells from experiments made or the penetration of dye from methylene blue solution into the vacuole, without determining the nature of the dye inside and outside the cell.

[Reprinted from The Journal of General Physiology, September 20, 1927, Vol. xi, No. 1, pp. 83–99.]

SOME ASPECTS OF BIOELECTRICAL PHENOMENA.*

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Accepted for publication, June 3, 1927.)

This brief sketch is intended as an introduction to a series of articles[1] on bioelectrical phenomena, its purpose being to present certain fundamental facts and underlying conceptions.

Early in the course of the investigation it became evident that there are great advantages in using single cells in place of tissues. The experiments were accordingly made with single (multinucleate) cells of *Valonia* and *Nitella*, which are large enough[2] to permit leading off simultaneously from several places on the same cell. This has important technical advantages and eliminates certain complications[3] which always arise in the study of tissues. In addition it enables us to find out to what extent changes in any part in the cell may affect other parts. A study of such effects and of their transmission in protoplasm may be expected to throw some light on the propagation of stimuli in general and on the constitution of living matter.

Another advantage attending the use of these cells may be mentioned here. The study of bioelectrical phenomena has been hampered because nothing could be measured except potential differences between selected spots, and it has been impossible to determine the

* Contribution from the Bermuda Biological Station for Research, No. 156.

[1] The author desires to express his gratitude to the Carnegie Institution of Washington, D. C., which made possible the beginning of these investigations.

[2] The cells of the marine alga *Valonia* reach a length of 2 inches or more and those of the fresh water *Nitella* a length of 5 inches or more. The cells consist of a thin layer of protoplasm (containing numerous chloroplasts and nuclei) outside of which lies the cell wall and inside of which is the very large central vacuole filled with cell sap.

[3] For example, in a tissue the circuit includes a number of cells between which is intercellular material of some sort. If a cell is injured cell sap comes out and alters the intercellular material which in turn alters the potential difference of the uninjured cells. With single cells this cannot occur.

absolute value of the potential difference across the protoplasm at any one point. Since it is highly desirable to obtain such absolute values an attempt was made to do so. It would not be possible to make such determinations in tissues or in cells of ordinary size, but the use of very large cells enables us to reach the desired end.

In the case of *Valonia* this was done by piercing the cell with a capillary glass tube filled with cell sap (Fig. 1). On leading off from the interior of this tube to the outside of the cell we obtain a circuit which passes only once through the protoplasm (as indicated by the dotted line), and hence the measured E.M.F. gives the potential difference across the protoplasm at any point where an external contact is applied. In many cases the protoplasm attaches itself to the capillary at F so as to form an electrical seal, thus preventing any short circuit through the wall (between F and G) and along the outside of the capillary into the sap, and only such cells were employed in the experiments.

In the case of *Nitella* the same purpose was accomplished by reducing the potential difference at one point approximately to zero, by killing the protoplasm in such fashion[4] as not to affect other points some distance away on the same cell (at least for some time). In leading off from the killed point to a normal region the circuit passed once through the killed spot and once through living protoplasm, and the results justify the conclusion that when the experiments are made under the proper conditions the observed electromotive force is practically all due to the potential difference across the living protoplasm at the selected point.

In order to interpret the results of our measurements we need information regarding the structure of the protoplasm. There is some evidence to show that in general the surface of protoplasm differs from its interior, and some experiments indicate that the surface is non-aqueous. The interior of the protoplasm may be an aqueous phase consisting of sol or gel or both, or it may be an emulsion in which the outer phase is aqueous. We might therefore, as a working hypothesis, consider the protoplasm to be made up of an aqueous phase, W, and phases which are probably non-aqueous, forming the

[4] This can be done in a variety of ways which will be discussed in detail in subsequent papers.

external (X) and internal (Y) surface. These layers may be very thin (possibly monomolecular), or if thicker they may consist of sol or of gel or of an emulsion, the outer phase of which is non-aqueous.

It should be emphasized that this conception is set up merely as a working hypothesis which may be useful for the time being (some new evidence for the existence of layers will be presented in later papers). It is quite possible that the boundary surfaces are aqueous in character, and if the protoplasm really consists of layers it is quite possible that there are more than three. For the present, however, we shall adhere to the hypothesis in the form presented above.

Let us now consider under what conditions bioelectric effects may be expected to arise. If the protoplasm is made up of layers it may, for convenience, be represented as in Fig. 1 (in which G represents a salt solution applied to the cell wall and quickly penetrating through it to the surface of the protoplasm). We shall discuss certain possibilities on the assumption that these layers exist. It will then be evident what conditions would obtain if the protoplasm were not made up of layers.

Let us first consider the cell wall. This is of cellulose, and the experiments show that it is readily permeable to salts; so that an applied salt solution quickly penetrates the cell wall and comes in contact with the external surface of the protoplasm. If the salt solution has ions which move at different rates in the cell wall a diffusion potential will be set up. This however would not last long if the salt diffused only at right angles to the surface since the cell wall is very thin and very permeable; but a potential difference due to diffusion along the wall (from G toward F) might last a long time, but this would have little or no effect on the E.M.F. in the cell, measured as shown in Fig. 1, since in the experiments only those cells were used in which the protoplasm had made an electrical seal[5] at F so that no current

[5] It is an easy matter to tell whether this seal is made. If we place 0.6 M KCl at G and lead off from G to a drop of 0.6 M KCl placed on the outside of the cell at F (*i.e.* at the point where the capillary enters) it is evident that if there is a leak around the capillary we shall get the same potential difference as if we led off from G to the interior of the capillary; when the seal is made we actually observe a very different value. We arrive at the same result if we first lead off as shown in Fig. 1 and then immerse the cell completely in the solution applied at G. This will be discussed in later papers.

could leak along the outside of the capillary; hence the wall did not form a short circuit between *F* and *G* and any potential difference due to the diffusion of solution in the wall from *G* toward *F* would probably have only a negligible effect on the measured E.M.F.

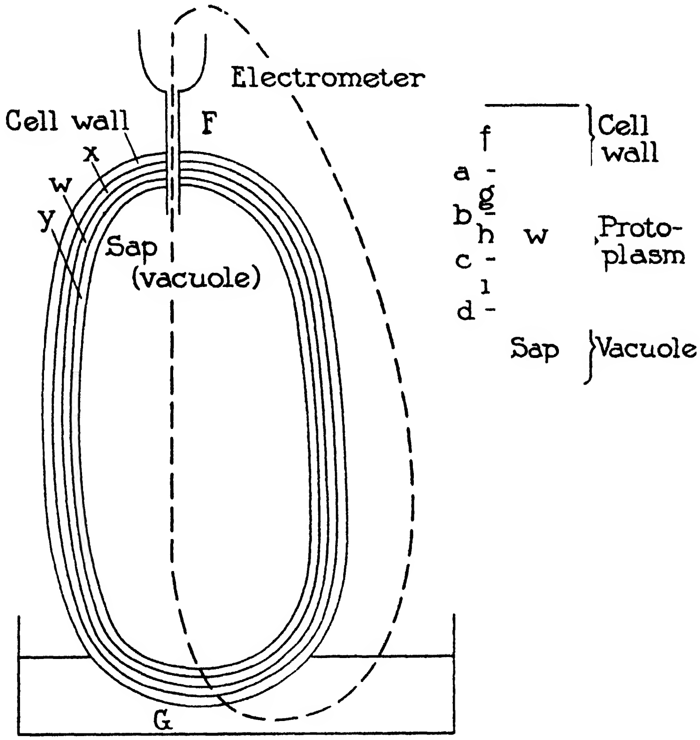

FIG. 1. Diagram of a cell of *Valonia* with inserted capillary. The thickness of the protoplasm and cell wall is exaggerated, being only a few microns, while that of the vacuole may be over an inch: *a*, *b*, *c*, and *d* are the seats of phase boundary potentials, and *f*, *g*, *h*, and *i* the seats of diffusion potentials. The circuit is supposed to follow the course of the dotted line.

What has been said about diffusion in the cell wall might apply to any of the other layers which are readily permeable to salts; but *X* and *Y* may be nearly or quite impermeable.

In addition to diffusion potentials we may consider phase boundary potentials, which may arise for example at *a*, *b*, *c*, and *d* (providing *X*, *W*, and *Y* represent distinct phases). The cell wall is here omitted from consideration since it appears too permeable to be the seat of phase boundary potentials.

The outer layer *X* is probably permeable to some extent to certain ions, which may give rise to potential differences when brought in contact with it: in this case current[6] must be able to pass through *X*.[7]

We must consider the possibility that *Y* may be almost or quite impermeable to ions, a possibility which is indicated by the situation in *Valonia*. Little or no Mg or SO_4 penetrates the vacuole, yet it seems probable that the protoplasm contains S and the chlorophyll bodies embedded in the protoplasm must contain Mg. It might therefore seem possible that Mg^{++} and SO_4^{--} penetrate *X* but not *Y* (unless $MgSO_4$ penetrates *X* only in the form of undissociated molecules). (It is also possible that the continuity of *X* is interrupted over each chlorophyll body so that Mg can gain access to it without passing through *X*.) If the layer *Y* is impermeable to ions generally, it is evident that the potential at both its surfaces, *i.e.* at *c* and *d* (Fig. 2), might under certain conditions be zero.

If *Y* were almost or quite impermeable to ions this would explain certain facts[8] which indicate that in general ions cannot penetrate readily into the vacuole. If we suppose that all the layers are permeable to ions we should assume that under normal con-

[6] The mere fact that E.M.F. produced at *B* and *C* can affect the measuring instrument does not prove that *X* conducts much current, since a very minute current can keep the electrometer charged, as can be shown by inserting a condenser in series with the cell.

[7] If *X* were aqueous it would of course conduct.

[8] This evidence has been gathered chiefly from studies by a number of investigators on the penetration of weak acids (for references see Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–27, viii, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255) and of bases, as well as of dyes (*cf.* Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561), which show that ions penetrate very slowly or not at all. The experiments of several investigators, especially unpublished results of Dr. Blinks, show that the resistance of the protoplasm is very high and unless this is due to polarization it must indicate a very low degree of permeability to ions on the part of some or all of the layers.

ditions this permeability is very slight. What has previously been said regarding the protoplasm applies especially to the marine alga *Valonia macrophysa*. Let us now consider the situation in the fresh water plant *Nitella*. In this case it is difficult to insert a capillary on account of the small size and the delicacy of the cells. We therefore perform the experiments by leading off from two places, as at *B* and *C*, Fig. 2. For convenience we shall postulate during the present discussion that the current flows chiefly in the circuit indicated by the dotted line. There may, however, be a short circuit in any layer. It seems probable that the only layer in which such

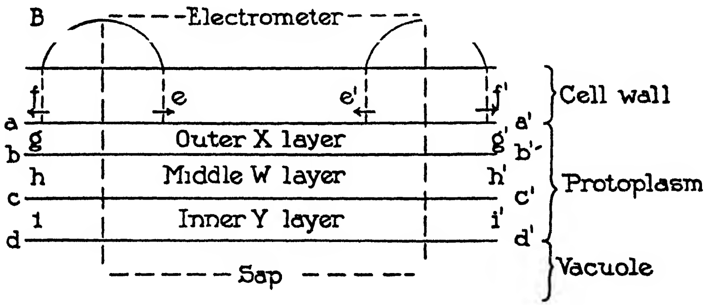

FIG. 2. Hypothetical diagram of a portion of a cell. *B* and *C* represent drops of solution applied to the exterior: *a*, *b*, *c*, and *d* represent the surfaces of the layers and are the seats of phase boundary potentials; *e*, *f*, *g*, *h*, and *i* represent the seats of diffusion potentials in the cell wall and in the layers of protoplasm. The main circuit is supposed to follow the course of the dotted line. The thickness of the cell wall and of the protoplasm is only a few microns.

short circuiting is important is the cell wall (the other layers being probably too thin or too resistant to permit much current to flow), and even in the cell wall this effect is apparently very small when it is imbibed with distilled water[9] or with tap water, as in *Nitella*. In *Valonia*, where the cell wall is imbibed with sea water, the short-circuiting effect would become important if the protoplasm did not attach itself to the capillary to form an electric seal (at *F*, Fig. 1)

[9] But the diffusion potentials in the cell wall due to the solutions at *B* and *C* might be greater in some cases when the wall was imbibed with distilled water than when imbibed with a salt solution.

which prevents short circuiting; this seal was secured in all the experiments.

If the solution applied at B differs from the solution with which the cell wall is imbibed, potentials may arise at e and f, which may set up "eddy" currents[10] flowing through the cell wall and through the protoplasm back to the drop applied at B. Their magnitude would depend on the potential differences as well as on the resistances involved. It is difficult to say what effect they would have upon the current which follows the course indicated by the dotted line in Fig. 2, but it seems probable that any effect will be of brief duration.[11] Similar "eddy" currents might be set up in any of the layers.

When identical solutions are placed on B and C it frequently happens that little or no potential difference is observed. Under these circumstances it seems reasonable to assume that the potential difference at a is equal and opposite to that at a', etc., and that diffusion potentials likewise cancel out. If the solutions applied to B and C in such cells are not identical it is probable that all the values except those at a and a' and at g and g' are equal and opposite. This would, of course, differ from the circuit in *Valonia*, as shown in Fig. 1. In other respects, however, what is said of *Valonia* applies to *Nitella*, and the following discussion applies to both.

Solutions applied to the surface will probably not affect the deeper layers for some time (unless they are very toxic solutions which break down or alter X), so that in brief experiments with non-toxic solutions we may consider that any observed changes depend only on the effect upon X and it will make no difference in the interpretation of the results whether we regard the protoplasm as consisting of one or of many layers. The hypothesis that there are several layers becomes important when we deal with toxic effects or other alterations in the protoplasm.

Polarization may, of course, be expected at any of the layers with a consequent diminution of the current.

The observed potential difference may be made up of the phase

[10] There is, of course, a current flowing from B to C through the cell wall and back through the galvanometer, as already mentioned.

[11] The experiments show that in general when one solution is substituted for another the observed changes are completed in a few seconds unless injury occurs.

boundary potentials at a, b, c, and d, and of the diffusion potentials in X, W, and Y. In addition there may be a diffusion potential in the cell wall, but this will be of short duration if it is due solely to diffusion across the wall, since the wall is very permeable: if it is due to diffusion along the wall it may last for some time.

A potential difference is usually observed when we lead off from B and C[12] (Fig. 2) with solutions of the same salt at different concentrations (concentration effect), or with solutions of different salts (chemical effect). In general we observe both effects with protoplasm but the experiments show that in the cell wall with the solutions thus far employed only the concentration effect is of importance. It is possible to arrange the experiments in such fashion that the effect due to the protoplasm can be ascertained, at least approximately. Throughout this paper the effects discussed are those on the protoplasm unless otherwise stated.

It is commonly observed that when a solution of KCl is applied at one point and a solution of NaCl of the same molar concentration at another point, KCl is negative to NaCl. How is this to be explained?

If we regard the whole effect as due to diffusion potential we may say that the mobility of K in the outer protoplasmic layer[13] (X, Fig. 1) is greater than that of Na. This is to be expected if the layer X behaves, for example, like phenol, as described by Nernst and Riesenfeld,[14] or like the collodion membranes studied by Michaelis and Perlzweig.[15]

The fact that a concentrated solution of KCl is negative to a dilute solution would mean that K penetrates more rapidly than Cl. This would leave the solution negatively charged, the effect being greater

[12] The corresponding experiment is performed with *Valonia*, as in Fig. 1, by leading off from G, first with one solution, then with the other, and taking the difference between the two measurements.

[13] In the brief experiments here referred to it is not probable that any of the deeper layers are involved since the potential differences with which we are here dealing are established within a few seconds. The cell wall appears to play little or no rôle in connection with the chemical effect.

[14] Nernst, W., and Riesenfeld, E. H., *Ann. Physik*, 1902, viii, series 4, 600.

[15] *Cf.* Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, x, 575, where references to earlier papers are given.

as the concentration increases: hence the concentrated solution would be negative to the dilute solution. Since in general in biological experiments dilute solutions of salts are usually positive to more concentrated solutions of the same salt, we might conclude that in general cations tend to penetrate more rapidly than anions (this interpretation would not necessarily hold if the potential differences were due to phase boundary potentials).

Let us now consider phase boundary potentials. The foundation of the theory of these potentials was laid by Nernst.[16] He assumes that the tendency to enter is not the same for all ions. Thus, let us suppose that we have to do with LiCl, and that the concentration of Li in the external solution is C_{Li} and that it tends to enter X and to reach the concentration $A_{\text{Li}}C_{\text{Li}}$ in X, A_{Li} being the "true" partition coefficient[17] of Li. The corresponding coefficient of Cl is A_{Cl}, and if this is less than A_{Li} (*i.e.* if Cl is less soluble in X than Li is) Li will be unable to reach its "true" value, since it cannot enter in excess of Cl (except perhaps at the very surface), but Cl will enter in excess of its "true" value. The actual concentrations reached in X may be called C'_{Li} and C'_{Cl} and these must be equal. Nernst shows that this leads to the equation

$$\text{P.D.} = RT \log \frac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}} = -RT \log \frac{C_{\text{Cl}}A_{\text{Cl}}}{C'_{\text{Cl}}}$$

Hence

$$\log \frac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}} = -\log \frac{C_{\text{Cl}}A_{\text{Cl}}}{C'_{\text{Cl}}} \text{ and } \frac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}} \quad \frac{C'_{\text{Cl}}}{C_{\text{Cl}}A_{\text{Cl}}}.$$

Multiplying both sides by $\dfrac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}}$ and substituting the values

$$C_{\text{Cl}} = C_{\text{Li}} \text{ and } C'_{\text{Li}} = C'_{\text{Cl}}, \text{ we obtain } \frac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}} = \sqrt{\frac{A_{\text{Li}}}{A_{\text{Cl}}}}.$$

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[16] Nernst, W., *Z. physik. Chem.*, 1892, ix, 140, Nernst, W., and Riesenfeld, E. H., *Ann. Physik*, 1902, viii, series 4, 600. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

[17] The "true" partition coefficient is that which would be observed if Li could enter unhindered by Cl: this would be the case if the "true" partition coefficients of Li and Cl were equal.

Hence

$$\text{P.D.} = RT \log \sqrt{\frac{A_{\text{Li}}}{A_{\text{Cl}}}} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}}.$$

Haber[18] arrives by a different route at a formula which reduces to the same thing. Haber's formula is

$$\text{P.D.} = RT \log \left(\frac{C_{\text{Li}}}{C'_{\text{Li}}}\right) (K_{\text{Li}})$$

where K_{Li} is the solution tension of an imaginary Li electrode in X divided by its solution tension in water. It is evident[19] that K_{Li} is equal to the A_{Li} of Nernst's formula.

Hence we may write

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}} = \frac{RT}{2} \log \frac{K_{\text{Li}}}{K_{\text{Cl}}}.$$

If we apply LiCl at one point and NaCl at another the E.M.F. will be

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}} - \frac{RT}{2} \log \frac{A_{\text{Na}}}{A_{\text{Cl}}}$$

$$= \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Na}}} = \frac{RT}{2} \log \frac{K_{\text{Li}}}{K_{\text{Na}}}.$$

Hence it is evident that the P.D. depends only on the difference in the "true" partition coefficients,[20] or the solution tensions, and that if

[18] Haber, F., *Ann. Physik*, 1908, xxvi, series 4, 927. Haber, F., and Klemensiewicz, Z., *Z. physik. Chem.*, 1909, lxvii, 385.

[19] *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i, pp. 186, 190.

[20] The formula of Nernst has been extended to solutions containing more than one salt by Michaelis, L., and Fujita, A., *Z. physik. Chem.*, 1924, cx, 270, and by Horovitz, K., *Z. physik. Chem.*, 1925, cxv, 424. Thus for a mixture of NaCl and KNO_3 in water the formula would be

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Na}} C_{\text{Na}} + A_{\text{K}} C_{\text{K}} + A_{\text{H}} C_{\text{H}}}{A_{\text{Cl}} C_{\text{Cl}} + A_{\text{NO}_3} C_{\text{NO}_3} + A_{\text{OH}} C_{\text{OH}}}.$$

Li has a greater tendency to enter than Na, LiCl will be negative to NaCl.[21]

That these formulæ do not account for the concentration effect may be made clear by an illustration. If we apply LiCl at one spot in the concentration C_1 and at another in the concentration C_2 we have at one place P.D. $= RT \log \left(\frac{C_{1\text{Li}}}{C'_{1\text{Li}}}\right) K_{\text{Li}}$ and at the other P.D. $= RT \log \frac{C_{2\text{Li}}}{C'_{2\text{Li}}} (K_{\text{Li}})$. The total P.D. will be the difference between these or

$$\text{P.D.} = RT \log \left(\frac{C_{1\text{Li}}}{C'_{1\text{Li}}}\right) - RT \log \left(\frac{C_{2\text{Li}}}{C'_{2\text{Li}}}\right) = RT \log \left(\frac{C_{1\text{Li}}}{C_{2\text{Li}}}\right)\left(\frac{C'_{2\text{Li}}}{C'_{1\text{Li}}}\right).$$

[21] In order to visualize the situation it may be convenient to assign fictitious values which satisfy the requirements. This may be done as follows:

		B		*C*	
In *X*	"True" or "ideal" concentration	Li = 100	Cl = 25	Na = 36	Cl = 25
	Actual concentration (= C')	Li = 50	Cl = 50	Na = 30	Cl = 30
In external solution.	Actual concentration (= C)	Li = 1	Cl = 1	Na = 1	Cl = 1

In this case LiCl is applied at B (concentration = 1) and NaCl (concentration = 1) at C: $a_{\text{Li}} = 100$, $a_{\text{Na}} = 36$, and $a_{\text{Cl}} = 25$. At B the P.D. $= RT \log 100/50$ and the positive current tends to flow from the external solution into X since the concentration of Li in X is only 50 and its tendency is to push in until the "true" value of 100 is reached; on the other hand Cl tends to leave X since its concentration is 50 and it tends to move out to attain its "true" value of 25, and in consequence the P.D. $= -RT \log 25/50$. Na at C acts in the same way as Li at B, but the P.D. $= \text{RT} \log 36/30$. The total P.D. will be found by subtracting that at C from that at B, or

$$\text{Total P.D.} = RT \log \frac{C_{\text{Li}}A_{\text{Li}}}{C'_{\text{Li}}} - RT \log \frac{C_{\text{Na}}A_{\text{Na}}}{C'_{\text{Na}}} = RT \log \left(\frac{100}{50}\right)\left(\frac{30}{36}\right) = RT \log \frac{5}{3}.$$

This is evidently equal to $RT \log \sqrt{\frac{A_{\text{Li}}}{A_{\text{Na}}}} = RT \log \sqrt{\frac{100}{36}} = RT \log \frac{10}{6} = RT \log \frac{5}{3}$ as above. In this instance A_{Li} and A_{Na} are for convenience put greater than unity, but in an actual case we should expect them to be very much less than unity.

We should expect $\frac{C_{1Li}}{C'_{1Li}}$ to equal $\frac{C_{2Li}}{C'_{2Li}}$ and $\frac{C_{1Li}}{C_{2Li}}$ to equal $\frac{C'_{1Li}}{C'_{2Li}}$, so that the P.D. would be zero. It is evident that this would be the case since we can write

$$\text{P.D.} = RT \log \sqrt{\frac{A_{Li}}{A_{Cl}}} - RT \log \sqrt{\frac{A_{Li}}{A_{Cl}}} = 0.$$

This gives no concentration effect.

According to Wosnessnensky[22] it is possible to account for the concentration effect by supposing that the partition coefficients of the ions are not constant but vary independently with the concentration. In this case $\frac{C_{1Li}}{C'_{1Li}}$ would not be equal to $\frac{C_{2Li}}{C'_{2Li}}$. If we use the formula of Nernst it is easy to show that the sign of the dilute solution will depend on the relation between A_{Li} and A_{Cl}. If we assume for convenience that A_{Li} is always greater than A_{Cl} and that the latter remains constant while the former varies with concentration it is a simple matter to demonstrate that when A_{Li} is greater in the concentrated than in the dilute solution the latter will be positive (and *vice versa*).

Michaelis[23] states that a concentration effect is possible when a second electrolyte is present.

Since Haber and Klemensiewicz[18] found a concentration effect with H^+ ions in the case of certain kinds of glass[24] they assumed that a small amount of water is present in the glass giving a constant concentration of H^+ and OH^- ions. In that case we should have inside the glass $C'_{1H} = C'_{2H}$ and the equation would become

$$\text{P.D.} = RT \log \frac{C_{1H}}{C_{2H}}$$

which would explain the concentration effect.

To account for the concentration effect of various non-aqueous

[22] Wosnessensky, S., *Z. physik. Chem.*, 1925, cxv, 405.

[23] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i, 205.

[24] According to Horovitz this is not equally true of all kinds of glass (see Footnote 28).

liquids Beutner[25] assumes that an approximately constant concentration of certain ions results from a chemical reaction. Thus if we suppose X to contain an organic acid HA in very small amounts, the reaction

$$\text{LiCl} + \text{H}A \rightleftharpoons \text{Li}A + \text{HCl}$$

might occur.[26] If X contained equal numbers of Li^+ and Cl^- ions there would be no resulting P.D., but if HCl is less dissociated in X than is LiA the number of Cl^- ions would be less and a P.D. would result, which according to Beutner could be calculated by means of the formula

$$\text{P.D.} = RT \log \frac{C_{1\text{Li}}}{C_{2\text{Li}}}.$$

In order to employ this formula it is necessary to assume that HA is present in such small amounts[27] that practically all of it is converted to LiA even when the cation is present in the external solution in exceedingly low concentrations. This would give an approximately constant concentration of Li^+ in X.

The scheme proposed by Beutner involves a number of assumptions, in part tacit, which cannot be discussed here. Some of these assumptions are of very doubtful validity.[26]

If Beutner's scheme[28] (as presented by Michaelis[29]) should be applied to a series of chlorides, A, B, C (of the same molar concentration) such that A is negative to B, and B is negative to C, it would be said that the cation of A tends to be taken up more than that of B (since A is nega-

[25] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

[26] Michaelis and Perlzweig have raised a serious objection to this assumption (*cf.* Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, x, 575). There are other serious objections to Beutner's scheme.

[27] It is assumed that A comes out into the water to a slight extent only.

[28] In applying the equations for phase boundary potential we do not assume that the cell has reached complete equilibrium with the exterior, since in a living growing cell this is not to be expected, but it is possible to assume that the penetrating substance very quickly reaches approximately the equilibrium concentration at the surface or just inside the surface of X, in which case we should have approximately the value demanded by the equations.

[29] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i, 191 ff. *Cf.* also Foot-note 26.

tive to B) and that of B tends to be taken up more than that of C. (This is on the assumption made by Beutner that diffusion potentials are negligible.) Hence we must suppose that the cations of A and B penetrate X even if that of C is unable to do so. In this way it might be possible to determine what ions[30] enter X.

The glass used by Haber and Klemensiewicz acted as a hydrogen electrode only, but Horovitz[31] has found glasses which can take up silver and other cations and act as reversible silver electrodes, etc. This reminds us of the behavior of protoplasm which can act as a reversible electrode for many kinds of ions. The question arises whether the theory[32] formulated by Horovitz for these glasses can be applied to protoplasm. It would require us to assume that the concentration of ions in X cannot exceed a certain constant value which is independent of the nature of the ions and that no anions enter except combined with cations in the form of molecules which cannot dissociate in X (this does not imply that the substances in question are not wholly dissociated in the external solution, since we may assume that ions combine at the surface of X to form molecules and so pass through X). We should have to assume that the cell gives out as many cations as it takes up, but it is of course possible that it can produce enough H^+ ions for this purpose.

The conclusions already drawn regarding the series A, B, C would remain unchanged on the basis of the scheme proposed by Horovitz, providing diffusion potentials are neglected; this however is not permissible, according to Horovitz, so that we cannot tell which cation tends to be taken up to a greater degree, but we can say in regard to the series A, B, C that the cation of A tends to be taken up more than that of B or else has a greater mobility in X (or that both statements are true). On either basis we should conclude that the cation of A is able to enter X.

[30] Whenever the entrance or taking up of ions is mentioned it is of course understood that effects may be produced by the exit of these ions.

[31] Horovitz, K., *Sitzungsber. Akad. Wissensch. Wien, Math.-naturw. Kl., 2a Abt.*, 1925, cxxxiv, 335; *Z. Physik*, 1923, xv, 369. Horovitz, K., and Zimmermann, J., *Sitzungsber. Akad. Wissensch. Wien, Math.-naturw. Kl., 2a Abt.*, 1925, cxxxiv, 355.

[32] This is still unpublished. I am indebted to Dr. Horovitz for the privilege of seeing his manuscript in advance and for discussion of the theories here considered.

Let us now consider the Donnan potential.[33] As already stated[28] it may be doubted whether any part of an actively growing cell can come into a condition of real equilibrium with its surroundings and it could not very well be in equilibrium with two different solutions applied at different places. The question arises whether an approximate local Donnan equilibrium might be set up at two different points in contact with different concentrations of the same salt, so that we could calculate the P.D. by means of the usual formula

$$\text{P.D.} = RT \log \frac{C_1}{C_2},$$

where C_1 is the concentration of a diffusible cation in the external solution and C_2 its concentration inside the membrane.

If this were the case we might expect a concentration effect which would fall off with increase of concentration (as is the case with protoplasm[34]). But, as has been pointed out by Michaelis,[35] we should not expect this to be as large as that observed in the cell. If such an effect exists it seems very doubtful whether it can be calculated in this way since there are disturbing factors, such as movement of water due to osmotic pressure, etc.

On the other hand, it is difficult to see how a chemical effect could arise since at equilibrium all the diffusible cations would be expected to behave alike. They might, of course, differ in speed of penetration or in activity, but it is a question to what extent a temporary chemical effect could arise in this way. If it exists we should still conclude that if A is negative to B (in the series mentioned above) it means that more cations of A are taken up.

[33] This is variously classified by different authors but may for convenience be placed in a separate category. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i. Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol*, 1926–27, x, 575.

[34] The (unpublished) formula proposed by Horovitz, as well as that employed by Beutner, would lead us to expect an increase in concentration effect (*i.e.* an increased increment in potential difference for a fivefold dilution) as the concentration increases from zero, but after a certain point is reached no further increase in the concentration effect would be expected. It is found both with protoplasm and with the organic liquids immiscible with water studied by Beutner that the concentration effect falls off as the concentration increases above a certain point.

[35] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

We cannot decide at present to what extent bioelectric effects are to be attributed to diffusion potentials, to phase boundary potentials, or to Donnan potentials. It seems probable that in most cases two or more of these act simultaneously. We can, however, arrive at certain conclusions regarding the penetration of ions, provided we adhere to any of the schemes discussed above. Regarding the series of chlorides A, B, C previously referred to, we can say that the cations of A and B must be able to penetrate. For on the basis of any of the hypotheses outlined above we can say that even if the cations of C cannot enter, those of B must go in in order that B may be negative to C. Conversely, if we have a series of K salts D, E, F (of the same concentration), with D positive to E and E positive to F, we can say that the anions of D and E penetrate even if those of F do not.

We can also say that where there is a concentration effect not due solely to the cell wall ions must be able to enter the protoplasm.

Let us now consider the possibility of measuring the absolute values of certain potential differences. It seems probable that in brief experiments the applied salt solution does not penetrate through X into the deeper layers, and that in consequence any changes observed are due to changes in X. Let us suppose that we lead off from two places, B and C, and measure the potential difference of C against B[12]. Since the potential difference of B is opposite to that of C in the circuit, we may write

$$\text{Observed P.D. of } C = (a_C + Z_C) - (a_B + Z_B),$$

where a_C is the absolute value of the potential difference at the surface of X (a, Fig. 2) at the point in contact with C, and Z_C is the sum of the remaining values in X and in the deeper layers (the values of a_B and Z_B have corresponding significance).

If at the point in contact with B anions and cations tend to enter X to about the same degree, the value of a_B may be negligibly small and we shall have

$$\text{Observed P.D. of } C = a_C + Z_C - Z_B.$$

If the values of Z are the same at all points in the cell (assuming that the applied salt solution has not yet penetrated through X) this reduces to

$$\text{Observed P.D. of } C = a_C.$$

In this case we might be able to approximate the absolute value of a_C.

If we obtain the absolute value of the potential difference across the protoplasm at C, and if, as before, we write P.D. $= a + Z$, it is evident that if the value at a is negligibly small we may be able to approximate the value of Z. If it should happen (a possibility suggested above) that neither anions nor cations enter Y, the potential difference at both c and d might be zero and we should be able to approximate the value of $b_C + g + h$.

It seems evident from what has been said that bioelectiical investigations may throw some light upon the structure and properties of protoplasm. An especial advantage of this method of study is that it enables us to detect and record changes which last only a fraction of a second. It may thus uncover important activities of the protoplasm which would otherwise escape observation on account of the crudity of our methods of observation. This will be fully discussed in later reports.

SUMMARY.

It is pointed out that there are great advantages in using single cells instead of tissues in the study of bioelectrical phenomena.

Certain bioelectrical phenomena are discussed in relation to the structure of protoplasm.

Under certain circumstances measurements of potential differences may enable us to determine what ions enter the protoplasm.

Under suitable conditions we are able to ascertain the potential differences across the protoplasm at single points, instead of being obliged merely to measure the differences between two points.

[Reprinted from The Journal of Experimental Medicine, June 1, 1927, Vol. xlv, No. 6, pp. 1057–1063.]

THE DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS FOLLOWING INHALATION OF PNEUMOCOCCI.

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(Received for publication, February 21, 1927.)

In a preceding paper (1) it has been shown that mice may acquire a high degree of active immunity following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the immunity developed during a series of exposures. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

It has already been shown that rabbits are susceptible to infection by inhalation of Type I pneumococci (2), and that an occasional rabbit may recover from pneumococcus septicemia. In the present paper are reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of virulent Type I pneumococci. The duration of active immunity and the length of time that agglutinins and protective antibodies persist in the serum will be dealt with in a subsequent paper.

Method.

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of virulent Type I pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal.

The presence of agglutinins was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of Pneumococcus Type I. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intra-

peritoneal injection of 0.001 cc. of pneumococcus culture, of which 0.000,001 cc. killed a normal mouse within 48 hours. The rabbit serum and culture were administered simultaneously.

TABLE I.

Relation of Development of Agglutinins to the Number of Exposures.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " showing agglutinins.	4	13	12	14	12	9	9	8	6	6
Per cent of rabbits showing agglutinins	2	12	13	22	25	25	26	25	24	26

TABLE II.

First Appearance and Titre of Agglutinins.

Following exposure	1	2	3	4	5	6	7	8	9	10
Rabbit 1	—	1:10								
2	—	1:10								
3	1:50	1:50								
4	1:50	1:100								
5	—	—	—	1:20						
6	—	—		1:20						
7	1:20	1:20	1:20	1:20						
8	—	—	1:50	1:50						
9	—	1:20	1:20	1:20	1:20					
10	1:50	1:100	1:100	1:100	1:100					
11	—	1:10	1:20	1:20	1:20					
12	—	—	1:10	1:10	1:10	1:10				
13	—	—	—	—	—	—	1:10			
14	—	1:10	1:40	1:40	1:100	1:100	1:100	1:100		
15	—	—	1:10	1:20	1:100	1:100	1:100	1:100		
16	—	1:40	1:40	1:50	1:50	1:50	1:100	1:100	1:100	1:100
17	—	1:10	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50
18	—	—	—	—	1:100	1:200	1:200	1:200	1:200	1:200
19	—	1:20	1:40	1:40	1:40	1:40	1:40	1:40	1:40	1:50
20	—	—	—	—	1:10	1:10	1:20	1:40	1:40	1:40
21	—	1:20	1:40	1:40	1:50	1:50	1:50	1:50	1:50	1:50

Development of Agglutinins.

Out of 231 rabbits sprayed from 1 to 10 times, 80 or 34 per cent died from pneumococcus septicemia. The greatest number of deaths occurred after the first exposure. The sera for the serological reactions

which form the basis of this paper, were obtained from rabbits which had not only the greatest natural resistance, but also a certain degree of immunity acquired as the result of repeated exposures to live pneumococci.

In Table I are shown the number of rabbits which were sprayed from 1 to 10 times, the number which died of pneumococcus septicemia, and the number whose serum showed agglutinins in dilutions of at least 1:10. From Table I it is seen that only 4 or 2 per cent of the 135 rabbits which survived the first exposure developed demonstrable agglutinins. The percentage of rabbits showing agglutinins rose after the 2nd spray to 12 per cent, increasing to 25 per cent on and after the 5th spray. In fact 17 rabbits, after having been sprayed 10 times, failed to develop demonstrable agglutinins.

In all, 21 rabbits developed agglutinins. In Table II are shown the exposure after which agglutinins first appeared and the titre of agglutinins following each subsequent spraying. Although 4 rabbits showed agglutinins after the first exposure, a second exposure was necessary to incite the formation of these antibodies in 9 others. 1 rabbit first showed agglutinins only after 7 exposures. Great variations are also seen in the antibody titre of the different animals. As a rule agglutinins were first demonstrable only in the higher concentrations of serum (1:10 or 1:20) but in one instance the reaction first appeared following the 5th spray and was then present in a dilution of 1:100. Following the initial appearance of agglutinins there is a tendency for the titre to rise after the next spray and then to remain stationary. Of 8 rabbits which were sprayed 8 times, only 3 showed an agglutinin titre of 1:100; the serum of 3 others showed a titre as high as 1:50, while in one exceptional instance the serum suddenly showed agglutinins after the 5th spray in a dilution of 1:100 increasing after the 6th spray to 1:200.

Protective Antibodies.

In order to determine whether the serum of normal rabbits contains any natural antibodies against Type I pneumococcus, 1 cc. of serum from each of 147 normal rabbits was injected intraperitoneally into as many mice. The next day these mice were injected intraperitoneally with 0.000,001 cc. of virulent culture of Type I pneumococcus.

136 or 92 per cent of these mice died. In order to determine whether the 11 surviving mice recovered by virtue of a natural antibody present in certain normal rabbit sera, or by reason of a non-specific reaction induced by foreign protein, plain broth and normal horse serum were tested. 25 mice received in like manner injections of 1 cc. of plain broth, and another 25 were injected with 1 cc. of normal horse serum. 24 hours later all 50 mice were inoculated intraperitoneally with 0.000,001 cc. of virulent Type I pneumococcus. Of the mice receiving a preliminary injection of broth 24 or 96 per cent died, and of those injected with horse serum 17 or 68 per cent succumbed to subsequent infection. From the results of this experiment it would seem that the mere prelim'nary injection of a foreign serum of either the horse or rabbit, in certain instances conferred protection in mice against an

TABLE III.

Number of Rabbits Exposed and Number Whose Serum Protected Mice against 0.001 Cc. of Pneumococcus Type I.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits .	135	108	87	63	48	36	34	31	25	23
" " " whose serum protected	5	10	14	25	22	22	21	23	19	19
Per cent of rabbits whose serum protected	3	9	16	39	45	61	61	74	76	82

otherwise fatal inoculation of Type I pneumococcus. From this it is evident that the 8 per cent of normal rabbit sera which afforded protection did so not because of the presence of natural antibodies but because of the non-specific protective reaction induced by the foreign serum.

In Table III are shown the number of rabbits which were sprayed from 1 to 10 times with Type I pneumococci and the number thus exposed whose serum subsequently protected mice against intraperitoneal injection of 0.0001 cc. of a virulent culture of the homologous organism. From this table it is seen that protective antibodies were demonstrable in the sera of 5 or 3 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits showing protection steadily increased, until after the 10th spraying the

serum of 82 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. A total of 49 rabbits developed protective antibodies. The spray following which the rabbit sera first protected mice is shown in Table IV.

From Table IV it is seen that with each successive spray the number of rabbits in whose sera protective antibodies were demonstrable progressively increased. Although the greatest number of rabbits showed protective antibodies in their sera after the 6th spray, other rabbits did not develop these antibodies until after the 10th exposure.

Correlation of Agglutinins and Protective Antibodies.

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentrations of 1:10,

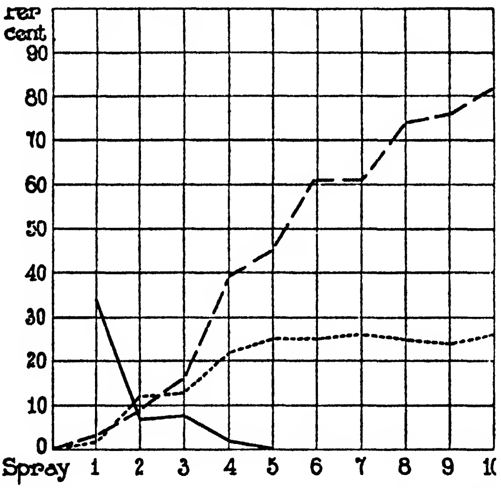
TABLE IV.

First Appearance of Protective Antibodies.

No. of spray	1	2	3	4	5	6	7	8	9	10
" " rabbits first showing protective antibodies	5	7	7	12	7	5	1	4	—	2

protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.0001 cc. of virulent pneumococcus culture. If smaller doses of Pneumococcus had been used, the presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of a high degree of immunity. In 10 instances both agglutinins and protective antibodies occurred after the same spray; in 9 animals agglutinins appeared before protective antibodies were demonstrable, while in 30 instances protective antibodies occurred without demonstrable agglutinins in the serum. In the 9 rabbits in which agglutinins appeared first, protective antibodies were later demonstrated in 4 instances after the next spraying, in 2 others after 3 additional sprayings, and in 1 rabbit not until after 5 subsequent exposures.

The relative occurrence of agglutinins and of protective antibodies in the serum of rabbits following inhalations of pneumococci is graphically shown in Text-fig. 1. In this figure the mortality curve of rabbits dying from septicemia is also given. It is seen that after the 1st exposure 34 per cent of rabbits died from pneumococcus septicemia. However, no rabbits died from this cause after the 4th spraying. In other words, the more highly susceptible animals were rapidly elimi-



TEXT-FIG. 1. Comparison of mortality, and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type I pneumococci.

——— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

------- per cent of rabbits showing agglutinins in serum following spraying.

— — — per cent of rabbits showing protective antibodies in their serum.

nated by the 1st exposure, while those that survived were either naturally more resistant or had gained some degree of immunity. It is interesting to note that up to the 5th spray an increasing proportion of rabbits developed agglutinins but that after this the percentage remained stationary. On the other hand, with each successive spray an increasing number of rabbits developed protective antibodies until

following the 10th exposure 82 per cent of the total number showed the presence of these immune substances in their serum.

DISCUSSION.

From the foregoing experiments it appears that following repeated inhalations of living Type I pneumococci, rabbits develop a high degree of immunity as evidenced by the presence of demonstrable agglutinins and protective antibodies in their serum. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may even recover from a transient pneumococcus septicemia. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which after implantation are able to invade the tissues, and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS.

1. Following repeated inhalations of Type I pneumococci agglutinins and protective antibodies can be demonstrated in the serum of rabbits.

2. The percentage of rabbits whose serum shows agglutinins remains stationary after the 5th exposure, but the percentage of rabbits showing protective antibodies in their sera steadily rises.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 1093–1106.]

STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

II. THE INFECTIVITY OF TYPE III PNEUMOCOCCUS FOR RABBITS.

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(Received for publication, February 8, 1927.)

In a previous paper (1) it was shown, in agreement with the experience of others, that the sera of rabbits immunized with Type III pneumococci failed to agglutinate the homologous organisms. Evidence was offered that the failure in agglutination was due, not to the inagglutinable state of the encapsulated bacteria, but to the actual absence of demonstrable type-specific antibodies. However, the sera of the immunized animals were reactive with pneumococcus nucleoprotein regardless of its type derivation and were capable of agglutinating the non-encapsulated degraded variants of all type-specific strains (R forms). This antibody response is contrary to the usual experience in immunization with type-specific pneumococci, when the cells used as antigen are intact. Although the Type III organisms used were, for the most part, possessed of large capsules, the antibody response, instead of being type-specific, was only species-specific in character and simulated that elicited by immunization with pneumococcus protein or with non-encapsulated R forms. The inference drawn from these results was that rabbits possess some mechanism which is capable of affecting the antigenic integrity of Type III pneumococci and that the alteration which the organisms undergo in the animal body is reflected in the character of the antibody response.

Because of the unusual reaction of rabbits to immunization with Type III pneumococci and the implications which these results suggest, investigations have been carried out with regard to the infectivity of this type of pneumococcus for rabbits. The experiments reported in the present paper include observations on the degree of viru-

lence of several strains of Type III, on the character of the bacteremia following intravenous injection, and the relation of phagocytosis to the disposal of the injected organisms.

Although Type III pneumococci are recognized as being highly pathogenic for white mice, reports on their virulence for rabbits have differed. Hanes (2) states that Type III organisms are highly virulent for rabbits without giving dosage or number of strains tested. Singer and Adler (3) found 0.05 to 0.1 cc. of culture usually fatal, but mention the fact that the lethal dose, in their experience, was not constant. They employed two strains and found one more constantly virulent than the other. Lévy-Bruhl (4) found the minimum lethal dose to be 1 cc. with two strains of Type III and greater than 1 cc. with two other strains. Bengtson (5) reported the lethal dose to vary from 0.1 to 1 cc.; whether different strains were tested or not is not stated.

In order to obtain further information concerning the virulence of Type III pneumococci for rabbits, eleven strains of this organism were collected. The strains were obtained from either blood or sputum of patients suffering from lobar pneumonia. All of the strains, which had been recently isolated were found on first injection to be highly virulent for mice. Other strains, which were taken from stock, were first passed through mice to enhance their virulence for these animals before being tested in rabbits. This was done in order to exclude from the cultures degraded R forms of pneumococci, which are non-encapsulated, non-type-specific, and avirulent. Reimann (6) has pointed out that a pneumococcus culture of low virulence may be one in which R forms predominate over type-specific, encapsulated S forms. According to this view, the repeated passage through mice of a culture containing both forms increases the proportion of S organisms until, with the acquisition of maximum virulence, the culture is theoretically composed entirely of type-specific, encapsulated pneumococci. Consequently, the cultures of Type III used for virulence tests in rabbits, by first being made highly pathogenic for mice, fulfilled this requirement.

Rabbits were injected either intravenously or intraperitoneally, with 12 to 14 hour cultures in doses ranging from 2 to 10 cc. It may be seen from Table I that eight of the strains did not produce fatal infection. In the case of two other strains rabbits died following injection of relatively large doses, but the cultures isolated from the

blood of these failed to kill others. Although no sustained effort was made to enhance the virulence of all the strains, most of them were injected several times. Of the eleven strains only one acquired definite virulence for rabbits, which, by repeated passage, finally produced a fatal infection in doses of 0.0001 cc. Even with this strain

TABLE I.

Virulence of Type III Pneumococcus for Rabbits.

Strain of Type III	Virulence for mice	Rabbit No	Dose of culture	Site of injection	Results
	cc.		*cc.*		
A	.000001	1	5	Intravenous	Survived
A	000001	2	2	Intraperitoneal	"
A	000001	3	2	"	Died—2 days
Blood culture Rabbit 3		4	2	"	Survived
" " "		5	5	"	"
M	000001	6	2	Intravenous	"
M	.000001	7	3	Intraperitoneal	"
M	000001	8	10	"	"
F	000001	9	5	"	Died—3 days
Blood culture Rabbit 9		10	5	"	Survived
F	000001	11	8	"	"
L	000001	12	2	Intravenous	"
L	.000001	13	5	Intraperitoneal	"
H	.000001	14	5	Intravenous	"
H	.000001	15	10	Intraperitoneal	"
E	000001	16	5	"	"
S	.000001	17	5	"	"
S	.000001	18	8	"	"
B3	.000001	19	5	"	"
B3	.000001	20	2	Intravenous	"
B4	.000001	21	5	"	"
B4	000001	22	5	Intraperitoneal	"
B2	.000001	23	5	"	"
PH*	.000001	24	5	"	Died—2 days

* This strain became highly virulent for rabbits.

the degree of virulence has not remained constant and has often shown evidence of attenuation when kept out of the animal body for several days. Moreover, differences in the natural resistance of individual rabbits to the same strain is, in part, responsible for variations in the degree of virulence.

From the results obtained it seems justifiable to conclude that Type III pneumococci possess only slight initial virulence for rabbits. Furthermore, although type specificity and encapsulation are necessary for the highest degree of mouse virulence, these characteristics are not, in themselves, sufficient to overcome the natural resistance possessed by rabbits.

The conclusion that Type III pneumococci are of low pathogenicity for rabbits was arrived at by the simple procedure of injecting organisms and accepting the ultimate survival or death of the animal as a criterion of virulence. This method, however, throws no light either on the duration and intensity of the infection, or upon the method of recovery, a fact which has been emphasized by Bull (7) in a report on some of the characteristics of streptococcal and pneumococcal bacteremia in rabbits. The technique employed by him consisted in making blood cultures intermittently from the peripheral veins after the injection of organisms. The results obtained by Bull and others (7–10) have demonstrated the reliability of the method. Consequently, since Type III pneumococci failed to produce fatal infections in rabbits, it seemed of interest to observe the course of the bacteremia following injection of these organisms. This was done by means of blood cultures taken at frequent intervals following the introduction of organisms into the circulation. The results are diagrammatically represented in the accompanying text-figures in which the number of colonies per unit of blood is plotted on the ordinates and the time interval, at which the culture was taken, is plotted on the abscissæ (Text-figs. 1, 2, and 3).

There were available for this study both S and R strains of Type III pneumococci. The eleven S strains were typical and biologically identical. They possessed large, easily demonstrable capsules; they grew on blood agar with the production of mucoid colonies; they were bile-soluble; they all reacted equally well in Type III antipneumococcus horse serum. They were pathogenic for humans, the source from which they were derived, and were all equally virulent for mice, killing in doses of 0.000001 cc. However, one of the S strains differed in that it was made virulent for rabbits by rabbit passage, whereas the others were not virulent for these animals in doses ranging from 2 to 10 cc. In addition to the type-specific S strains of pneumococci, non-

type-specific, degraded R forms were used for comparative study. As described by Reimann (6) R forms may be obtained by cultivating type-specific organisms in homologous immune serum. Those used in this experiment were derived from a culture of Type III. Doses of 1 cc. failed to kill mice and doses of 10 cc. failed to kill rabbits; higher doses were not tested. The strains of pneumococci used in the present experiment, then, comprised representatives of each of the three varieties:

1. S strains of Type III pneumococcus; *virulent* for rabbits (designated SV). One strain belonged to this group.
2. S strains of Type III pneumococcus; *avirulent* for rabbits (designated SA). Ten strains belonged to this group.
3. R strains representing the degraded, non-encapsulated, avirulent variants of Type III pneumococci. Bacteria of this character are comparable to non-pathogenic saprophytes.

EXPERIMENTAL.

Cultures.—12 to 14 hour plain broth cultures in standard doses of 2 cc. each were injected intravenously regardless of the strain used. The actual number of organisms per cc. was not determined but under uniform conditions, it may be considered comparable for all the strains.

Blood Culture Technique—The technique employed varied only in minor details from that described by Bull (7) and was as follows: The organisms were introduced into the marginal vein of one ear of the rabbit and cultures were obtained from the opposite ear. Before making the culture, the ear was closely shaved along the marginal vein and then saturated with 95 per cent alcohol. The alcohol was wiped off with a dry sterile sponge and the vein slit transversely with a razor blade. The blood was allowed to drip perpendicularly from the edge of the ear and, after discarding the first few drops, 6 drops were collected in melted agar kept at 42° to 45°C. The melted agar and blood were mixed rapidly and poured into a Petri dish The plates were incubated for 36 hours and the number of colonies per plate recorded. Cultures were usually taken 15, 30, and 60 minutes after injection of the organisms, then at 2 hour intervals for 12 hours; after this, 2 or 3 times daily until the animal succumbed or permanent sterility occurred. After the first 12 hours subsequent cultures were taken from blood derived from a fresh slit distal to the previous cuts. This precaution was taken in order to obviate the possibility of organisms resident in the tissues at the site of the previous injury being washed into the agar by the flowing blood.

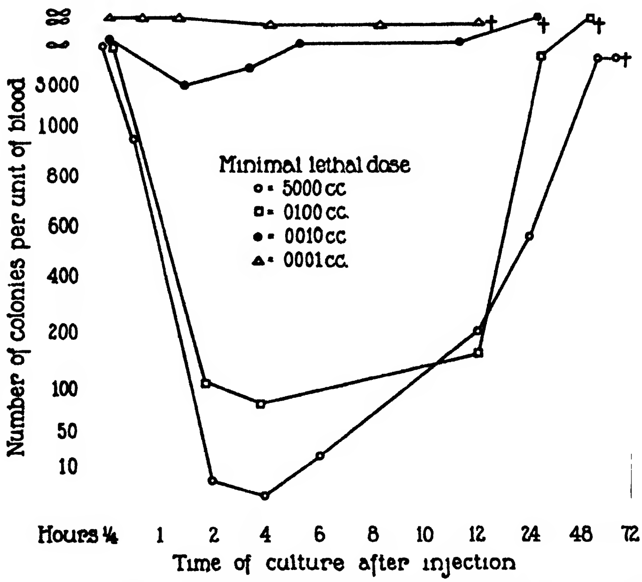
Employing streptococci and pneumococci Bull (7) found that infection in rabbits may take one of three courses depending on the viru-

lence of the bacteria. With highly virulent organisms a rapidly fatal septicemia occurred which was characterized by an initial marked reduction in the number of organisms in the circulating blood, followed by a rapid increase. With less virulent organisms the infection became chronic, the course of the bacteremia was uneven, and, after death, localization of organisms in the serous cavities was often demonstrable. When bacteria of even lower virulence were injected, they quickly disappeared and the blood stream remained sterile. The initial diminution in the number of organisms occurring in experimental septicemia, first described by von Fodor (8), has been repeatedly observed by others. This phenomenon has usually been explained as being due to the dissemination and filtration of the organisms throughout the body tissues. However, that this explanation is not sufficient becomes evident, as will be shown, if the relative virulence of the bacteria injected is taken into consideration.

Course of Bacteremia Following Intravenous Injection of Rabbit Virulent, S Strain of Type III Pneumococcus (SV Strain).

The effect of increased virulence on the course of the bacteremia is shown in Text-fig. 1. These results were obtained by injecting rabbits with a strain of Type III pneumococcus, the virulence of which had been progressively increased by repeated passages through rabbits. The data presented reveal differences in the course of the bacteremia depending on the degree of virulence. When the minimal lethal dose of the rabbit virulent strain (SV) was 0.5 cc. there was a marked initial decrease in the number of organisms in the circulating blood, the minimum occurring 4 hours after infection; then there followed a progressive rise in the number of colonies until the death of the animal occurred 56 hours after injection. This type of curve in experimental septicemia corresponds to the results previously described by others. When the virulence of this same strain had been enhanced so that now, 0.01 cc. of culture proved fatal, the initial decrease was less marked, and the secondary rise more rapid. Injection of a culture of still greater virulence (0.001 cc.) resulted in only a slight initial decrease in the number of organisms in the peripheral blood. When a maximum virulence of 0.0001 cc. had been attained the number of organisms in the blood of the infected rabbit was at no

time decreased and death resulted in 12 hours. In each instance approximately the same number of organisms was injected. Therefore, if the primary decrease represented merely a mechanical process of dissemination and filtration it should occur regularly, regardless of virulence. This, however, was not the case; the extent of the initial decrease in the number of circulating bacteria was in inverse proportion to the degree of virulence (Text-fig. 1).

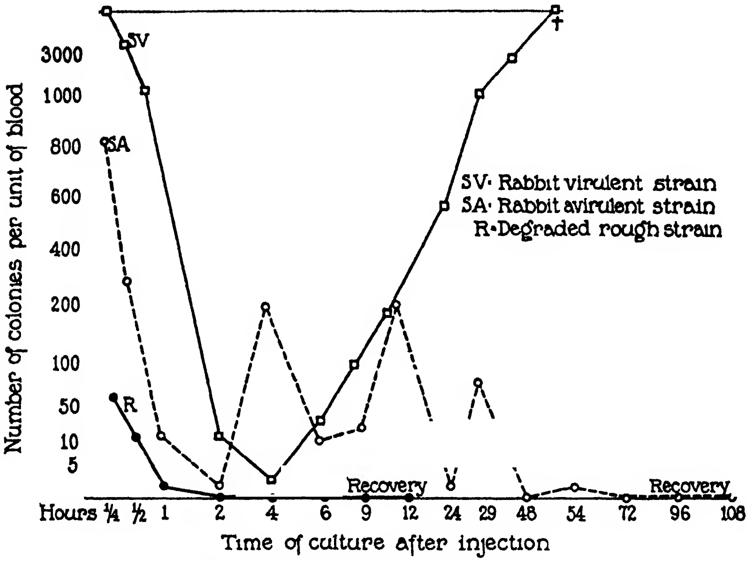


TEXT-FIG. 1. Effect of increased virulence on the course of bacteremia in rabbits injected with Pneumococcus Type III (Strain SV).

In addition to the effect of progressively increased virulence on the course of blood infection caused by the rabbit virulent strain of pneumococcus, certain characteristic differences in the curves were observed when strains of Type III not virulent for rabbits and degraded R forms of pneumococcus were employed. A graphic representation of the course of the bacteremia following injection of organisms of these two varieties is shown in Text-fig. 2. For purposes of comparison the type of curve representing the course of events after injection of the rabbit virulent strain is included in this same experiment.

Course of Bacteremia Following Intravenous Injection of R Strains of Pneumococcus.

From Text-fig. 2 it may be seen that there was an immediate and marked decrease in the number of R organisms in the circulating blood. After 2 hours they completely disappeared and the rabbit remained free from further blood infection.

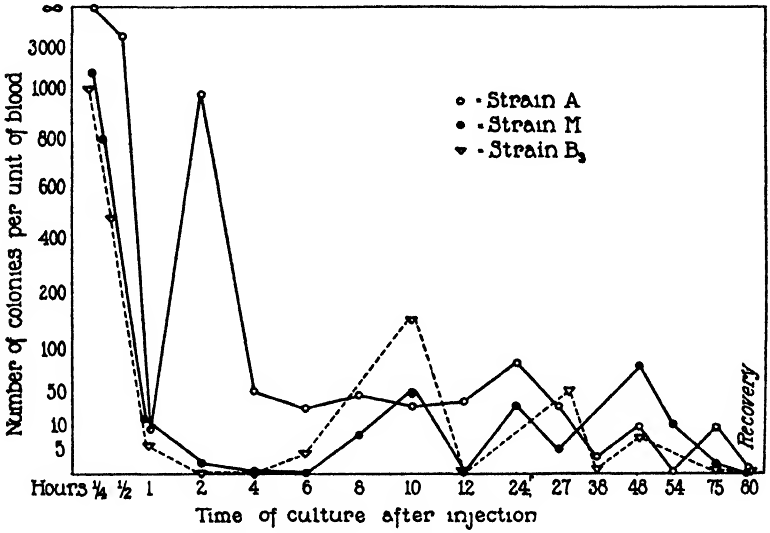


TEXT-FIG. 2. Course of bacteremia in rabbits injected with Pneumococcus Type III (Strains SV, SA, R).

Course of Bacteremia Following Intravenous Injection of Rabbit Avirulent, S Strains of Type III Pneumococcus (SA Strains).

The bacteremia which followed injection of rabbit avirulent strains of Type III pneumococci ran a distinctly different course. From Text-fig. 2 it may be seen that 3 to 4 days elapsed before permanent sterility of the blood was finally attained. While the bacteremia persisted, the chief characteristic was repeated fluctuations in the number of organisms in the circulating blood. At times the cocci entirely disappeared only to reappear again a few hours later. Exami-

nation of the organisms which reappeared failed to reveal any biological differences, when compared with the original cultures. As previously stated, the rabbit avirulent strains (SA) represent all but one of those used in the experiments. Although only one curve characteristic of the bacteremia produced by these strains is shown in Text-fig. 2, repetitions of this experiment gave similar results. In Text-fig. 3 are shown similar curves obtained with three other rabbit avirulent strains.



TEXT-FIG 3. Course of bacteremia in rabbits injected with strains of Pneumococcus Type III, avirulent for rabbits (Strains SA).

In the experiments just described virulence of Type III pneumococci for rabbits has been considered with special reference to certain properties pertaining to the organisms themselves, such as differences of encapsulation, type specificity, and mouse virulence. These factors have been correlated with the infectivity of the organisms as represented, not only by the ultimate outcome of the infection in rabbits, but also by the character of the bacteremia produced.

In addition to the biological properties of the bacteria, factors related to the resistance of the host require consideration and an investigation of two of these has been carried out.

1. Antibodies.—The sera of normal rabbits have not been found to possess antibodies reactive with the encapsulated Type III cells, or with the soluble specific substance derived from these organisms, or to confer passive protection on mice. Although normal rabbit sera are occasionally encountered which agglutinate R pneumococci, the incidence is not common and the dilutions of serum in which the reaction occurs are low. Whether antibodies of this character are significant in the disposal of R cells after injection into the animal body, is irrelevant to the present study. It can be stated, however, that the phagocytosis of R pneumococci, as described below, is not preceded by agglutination.

2. Phagocytosis.—In studying phagocytosis, the vital staining method described by Sabin (11) for the study of living blood cells has been employed. The technique varied in no detail from Sabin's procedure except for the fact that a small loopful of living pneumococcus culture was added to the blood. The preparations were observed microscopically in a warm chamber kept at 37°C. By this method leucocytes may be watched immediately after removal from the animal body and while actively motile in the environment of whole blood. Preparations of this character approximate conditions existing in the circulating blood and minimize alterations of the leucocytes which may occur in the usual methods of studying phagocytosis *in vitro*. The specimens were observed from 1 to 2 hours At the end of this time the leucocytes begin to lose their motility and their protoplasm contains large, red staining bodies which Sabin has described as vacuoles. Observations beyond 1 to 2 hours have not revealed phagocytosis which was not present earlier, and the beginning alteration in the leucocytes was evidence that the preparations were not useful for further observation.

When R forms of pneumococci were employed, phagocytosis by polymorphonuclear leucocytes could be seen in 2 to 3 minutes. It continued actively and, after 10 minutes, every leucocyte was engorged with organisms. When phagocytosis is observed under these conditions of vital staining the picture is very striking. Pneumococci, before ingestion, are unstained. However, immediately after being engulfed, they appear as bright red organisms within the body of the leucocyte. The facility with which R cells are phagocyted is

evidence of the prime importance of this activity in the natural resistance of rabbits to blood infection with R forms; the promptness with which these organisms disappear from the blood stream following intravenous injection may be referable, in part at least, to their susceptibility to phagocytosis.

When S forms of Type III pneumococci were mixed with normal rabbit blood, no phagocytosis was observed. This was true whether the encapsulated organisms were virulent or avirulent for rabbits. Occasionally, after an hour a few leucocytes could be found which had ingested one or two bacteria but the picture contrasted sharply with that seen in preparations made with R forms. Since the inability of leucocytes to ingest encapsulated bacteria has been a common observation, and, since this fact has been related to virulence, the failure to demonstrate this correlation in the present study is of special significance. Although phagocytosis as observed *in vitro* may not be identical with the phenomenon *in vivo*, nevertheless, the contrast between the action of leucocytes against R and S forms is striking and justifies the inference that the natural resistance of rabbits to S forms of Type III pneumococci involves factors either additional to or different from phagocytosis.

DISCUSSION.

In the experiments reported in this paper on the infectivity of Type III pneumococci for rabbits certain factors which are known to be associated with virulence and resistance have been taken into consideration. The organisms used in the experiments conformed with certain requirements which might be expected to promote virulence. All of the strains possessed large mucoid capsules and type specificity (S forms). They were obtained from patients suffering from lobar pneumonia and were highly virulent for mice. However, in spite of this, ten out of eleven of these strains failed to produce fatal infection in rabbits when injected in moderately large doses. The other strain was slightly virulent for rabbits on isolation and this property was further enhanced by animal passage. Since no biological differences could be demonstrated between this strain, when possessed of maximum virulence, and the others, it is necessary to assume that viru-

lence in this instance is related to some property not possessed by the other S strains. The course of the fatal septicemia resulting from injection of this strain and alterations in the character of the curve associated with increased virulence are shown in Text-fig. 1.

An attempt to understand the failure of *avirulent*, *encapsulated* Type III pneumococci (S forms) to cause fatal infection in rabbits led to a study of the fate of these organisms as contrasted with that of *avirulent*, *non-encapsulated* pneumococci (R forms). Although the ultimate survival of the infected animal occurs in both instances, the bacteremia induced by S forms differs from that induced by R forms. Following injection, the non-encapsulated R cells disappear rapidly and permanently from the blood stream, whereas the avirulent, but encapsulated S forms give rise to a prolonged bacteremia characterized by intermittent increase and reduction in the number of circulating organisms. These differences suggest that the mechanism whereby rabbits overcome the two types of infection is not identical, and that the method of disposal is in some way related to the presence or absence of capsule. Further evidence that the mechanism of recovery in the case of S organisms is different from that effective in disposing of R forms, was brought out in the experiments on phagocytosis. Therefore, it seems obvious that, although the encapsulated state is sufficient to prevent phagocytosis, it is not sufficient to protect the cell against the defense reactions of the host. The resistance of rabbits to Type III pneumococci implies the presence of factors other than phagocytosis. Furthermore, the fact that the sera of normal rabbits do not contain demonstrable type-specific antibodies renders improbable the participation of these immune substances in the mechanism of natural resistance. In a previous paper (1) it was shown that immunization of rabbits with Type III pneumococci failed, in the majority of instances, to stimulate the production of type-specific antibodies, but was effective in producing antibodies reactive against another constituent of pneumococci, namely the nucleoprotein fraction. These experiments indicate that normal rabbits possess a mechanism which is capable of inflicting an injury on the capsule of Type III pneumococci. It has been shown by Avery and Heidelberger (12) that type-specific antibodies are best elicited when S cells in an intact state are used as antigen. They (12) have also

shown that type specificity is intimately related to the soluble specific substance of the capsule of pneumococcus. Therefore, the absence of demonstrable type-specific antibodies in rabbits immunized with encapsulated Type III pneumococci indicates that the animals are capable of damaging the capsular mechanism of these organisms. In the present experiments, the capacity of normal rabbits to inflict injury on Type III pneumococci is further demonstrated by the recovery of the animals following intravenous injection of living S organisms. It seems not unlikely that both the survival of the animal and the altered antigenicity of the cell following injection of the encapsulated organisms are referable to the same mechanism, and upon the factors underlying this defense reaction depends the natural resistance of rabbits to Type III pneumococci.

SUMMARY.

The observations recorded in this paper on the infectivity of Type III pneumococci for rabbits may be summarized as follows:

1. Of eleven strains of Type III isolated from human sources, ten were found to possess low virulence for rabbits. This was true despite the fact that all the strains tested possessed large capsules and a high degree of virulence for mice.

2. One strain of Type III pneumococcus was rendered highly virulent for rabbits. Since it possessed no other biological property demonstrably different from the other strains, its virulence must reside in some additional property.

3. An initial decrease in the number of circulating organisms following the injection of virulent bacteria is a well known occurrence, and it was observed in rabbits injected with the rabbit virulent strain of Type III. However, the extent of the reduction was in inverse proportion to the degree of virulence of the strain; a fact which makes mechanical explanations of the phenomenon insufficient.

4. The bacteremia produced in rabbits by Type III pneumococci, avirulent for this species, runs a characteristic course. It differs from that produced by non-encapsulated R forms of pneumococci although in both instances survival of the infected animal ensues. This is evidence that the mechanism of resistance against encapsulated and non-encapsulated pneumococci is not identical.

5. Phagocytosis of Type III pneumococci by circulating rabbit leucocytes was not demonstrable by a vital stain technique, whereas under the same conditions the ingestion of non-encapsulated R forms occurred. This is further evidence that the process whereby non-encapsulated pneumococci are disposed of, is insufficient to explain the natural resistance of rabbits to infection with encapsulated Type III pneumococci.

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[Reprinted from The Journal of Experimental Medicine, August 1, 1927, Vol. xlvi, No. 2, pp. 343–356.]

STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

III. Increased Resistance to Type III Infection Induced in Rabbits by Immunization with R and S Forms of Pneumococcus.

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(Received for publication, May 26, 1927.)

In two preceding papers (1, 2) certain phenomena concerning the antigenicity and infectivity of Type III pneumococci in rabbits were described. The results of these experiments may be summarized as follows: Immunization of rabbits with Pneumococcus Type III (1) fails, in the great majority of instances, to stimulate the production of type-specific antibodies, but is always effective in eliciting antibodies reactive with pneumococcus nucleoprotein and R strains derived from all types of pneumococci. These results were interpreted as indicating that normal rabbits possess some mechanism whereby, following the introduction of Type III organisms into the animal body, the antigenic complex of the bacterial cell is so altered that the type-specific component is rendered ineffective as antigen. Since Avery and Heidelberger (3) have shown that type specificity resides in the soluble specific substance which is predominantly present in the capsule, it appears that this altered antigenicity is the result of an injury inflicted upon the capsular substance of the cell. Further evidence in support of this view lies in the fact that Pneumococcus Type III possesses low pathogenicity for rabbits. Ten out of eleven strains of this organism, although highly pathogenic for white mice and possessed of large mucoid capsules (S forms), were found to be avirulent for rabbits in doses of 2 to 5 cc. and sometimes 10 cc. (2). Since encapsulation and virulence are generally considered as being intimately associated, it seems possible that the method, whereby

rabbits resist Type III infection, rests on the same mechanism which is responsible for the destruction of the type-specific antigenicity of the cell and that by virtue of the injury inflicted on the capsule of the living organism, virulence is impaired. These results expressed in terms of the host imply that rabbits possess a considerable degree of natural resistance to Type III infection. However, to attribute resistance to natural immunity is merely restating the problem and leaves unexplained the mechanism in the body on which it depends. In the recovery of normal rabbits from Type III infection the evidence indicates that in this instance, the factors underlying resistance are primarily operative against the capsular component of the cell.

The results previously referred to have concerned the reaction of normal rabbits. The experiments reported in this communication deal with immunized rabbits, and the purpose has been to determine the presence of active immunity against Type III infection following immunization not only with homologous organisms but also with heterologous strains, both S and R forms, and with solutions of pneumococci. Others (7, 8) have reported that rabbits immunized with Pneumococcus Type III are actively immune against infection with homologous organisms even though specific agglutinins are not demonstrable. These results minimize the rôle which specific antibodies play in active resistance in the case of Type III infection, and Singer and Adler (8) have offered an explanation of this phenomenon to which subsequent reference will be made. It seemed of significance to determine if any demonstrable antibody participated in this active immunity; consequently the sera of all the experimental animals have been tested for both type-specific (anti-S), and species-specific (anti-P), antibodies and the results correlated with the presence or absence of effective resistance.

Testing for the presence of active immunity necessitates the use of virulent organisms. Although most of the Type III strains have been found to possess low initial virulence for normal rabbits, one strain was made highly pathogenic by rapid animal passage. Consequently this rabbit virulent strain afforded a means of testing for increased resistance. Its virulence was maintained so that .001 cc. always proved fatal and, in most of the experiments, .0001 cc. caused death. Since a standard dose of 1 cc. of culture was always injected,

each animal may be considered as receiving usually 10,000 and always 1000 lethal doses.

Methods.

Antigens.—Heat-killed cultures, regardless of the type or strain employed in immunization, were, in each instance, made by the same method. 12 to 14 hour plain broth cultures, killed by heating at 56° for ½ hour, were centrifuged and resuspended in physiological salt solution in such quantities that 0 5 cc. of the vaccine was equivalent to 1 cc. of original culture

The pneumococcus solutions used for immunization were made by two different methods One solution consisted of nucleoprotein derived from Type II pneumococcus according to the method described by Avery and Morgan (4). The other solution was made as follows: 6 liters of R_2 culture (derived from Type II pneumococcus) were centrifuged and the bacteria resuspended in 100 cc. of physiological salt solution. 0 3 cc of 10 per cent sodium desoxycholate was added, and the mixture incubated at 37 5°C. for 2 hours At the end of this time stained films of the fluid showed a complete dissolution of all formed cells. The solution was then centrifuged at high speed to remove detritus, and the supernatant fluid filtered through a Berkefeld V filter. The bacteria-free filtrate was used for immunization.

Methods of Immunization—All rabbits receiving heat-killed organisms were immunized according to the method described by Cole and Moore (5), which consists in alternating for 6 weeks, a week of daily injections of 0 5 cc of vaccine followed by a week of rest.

Rabbits injected with pneumococcus solutions received 0.5 cc. intravenously each day for the 1st week and during the 2 other alternate weeks of injection received 1 cc. daily.

All animals were bled 8 to 10 days after the last injection and the antibody content of the sera determined.

Method of Testing Active Immunity—As previously stated, a standard dose of 1 cc. of culture of the rabbit virulent strain of Type III was intravenously injected in each test animal. .0001 cc. of this culture was usually fatal and .001 cc. always killed normal rabbits.

For purposes of following the course of the blood infection in both immunized animals and normal controls, blood cultures were taken at frequent intervals according to the method previously used (2). 4 to 6 rabbits in addition to 1 or 2 controls were tested simultaneously.

Most of the animals which died were examined post mortem with special reference to gross pathological changes in the serous cavities.

Altogether, 44 rabbits have been tested for the presence of active immunity against infection with a rabbit virulent strain of Pneumo-

coccus Type III. In addition to animals which, by surviving, demonstrated a solid immunity, others were considered as partially immune, which, although ultimately succumbing, showed evidence of increased resistance by reason of the duration of life, the character of the bacteremia as contrasted with controls, and the presence of localized infection found post mortem. A more detailed consideration of these factors will be given in the analysis of the results.

The experimental animals, depending upon the pneumococcus material used for immunization, may be grouped as follows:

I. Rabbits immunized with Pneumococcus, Type III.
II. Rabbits immunized with Pneumococcus, Type I or II.
III. Rabbits immunized with Pneumococcus, R strains.
IV. Rabbits immunized with solutions of Pneumococcus.

Group I. Rabbits Immunized with Type III Pneumococcus.

Twelve rabbits belong to this group. They were chosen from the 28 rabbits used in the immunization experiments previously reported (1). The strain of Type III used for immunization of these animals, although encapsulated, type-specific, and highly pathogenic for mice, was avirulent for rabbits in doses of 5 cc. 3 of the 12 possessed type-specific agglutinins in low titre (2 of the sera were not reactive beyond 1:2 dilution and the other was not reactive beyond 1:20 dilution). The sera of the other 9 contained no demonstrable type-specific antibodies. The sera of all the animals possessed antiprotein antibodies, and agglutinated R strains in 1:160 or 1:320 dilutions of serum.

Of the 3 rabbits whose sera possessed demonstrable type-specific antibodies, 1 survived, 1 lived 7 days and showed evidence of increased resistance, 1 died simultaneously with the control. Of the other 9 rabbits, 5 survived, 3 lived 11, 16, and 22 days respectively showing evidence of increased resistance, 1 died with the control.

In summary, it may be seen that of the 12 rabbits immunized with Type III pneumococcus, 6 survived, 4 showed evidence of increased resistance, and 2 died at the same time as the controls. These results expressed in percentage are: 50 per cent survived and, by including the partially immune, 83 per cent showed definite evidence of increased resistance.

Group II. Rabbits Immunized with Type I or Type II Pneumococci.

Fifteen rabbits belong to this group; 10 were immunized with Type II, 5 with Type I, and all were subsequently infected with Type III. The sera of these animals contained homologous type-specific agglutinins to the usual degree and also antiprotein antibodies agglutinating R pneumococci to approximately 1:160 dilution of serum. Of the 10 rabbits immunized with Type II, 7 survived, 2 living 6 and 7 days respectively, were considered as possessing some immunity, 1 was unprotected. Of the 5 rabbits immunized with Type I, 4 survived and 1 was unprotected. The results obtained with rabbits immunized with heterologous type-specific pneumococci may be summarized as follows: 11 survived infection with Type III, and 2 showed evidence of increased resistance. Expressed in percentage, 73 per cent survived and a total of 86 per cent showed evidence of active immunity.

Group III. Rabbits Immunized with R Forms of Pneumococci.

Eleven rabbits belong to this group. 5 were immunized with an R strain derived from Type I pneumococcus (designated R_1); 5 with an R strain derived from Type II (designated R_2); 1 with an R strain derived from Type III (designated R_3). The sera of these rabbits contained no type-specific antibodies, but only antiprotein antibodies reactive with R strains in dilutions of 1:160 to 1:320. Of the 5 rabbits immunized with R_1, 3 survived, 1 lived 6 days with evidence of resistance, and 1, living 4 days, was classified as not being immune. Of the 5 immunized with R_2, 4 survived, and 1 lived 5 days with evidence of resistance. The 1 animal immunized with R_3 survived. The results with the rabbits immunized with non-type-specific, non-encapsulated R strains show that 8 survived, 2 showed evidence of increased resistance, and 1 was not considered as immune. Expressed in percentage, 72 per cent survived and a total of 90 per cent showed evidence of increased resistance.

Group IV. Rabbits Immunized with Solutions of Pneumococci.

Six rabbits belong to this group. The sera of these animals contained only antiprotein antibodies. The titre of R agglutinins obtained by immunization with purified nucleoprotein (Rabbits 24 and

25 in Table III) was 1:100. The antiprotein antibodies present in the sera of the rabbits immunized with desoxycholate solution of pneumococci (Rabbits 27, 28, 29, and 30) agglutinated R organisms in dilutions of 1:1260. No type-specific antibodies were demonstrable.

None of these animals showed evidence of increased resistance to the amount of culture with which they were infected. Although 1 lived $3\frac{1}{2}$ days, blood cultures showed countless organisms (∞) in each culture taken later than 18 hours after infection. Table III shows the course of the bacteremia in this group of animals, which is in striking contrast to the blood infection occurring in resistant animals (Tables I and II).

Analysis of Results.—The data presented in the foregoing experiments reveal the interesting fact that of 38 rabbits which had been previously immunized with pneumococci 25 survived the intravenous injection of living, virulent Type III organisms in amounts at least a thousandfold greater than the dose invariably fatal for normal controls. A second point of equal interest is the fact that this solid immunity against Type III infection may be established by preliminary treatment with cells of heterologous types and with the degraded R forms of pneumococci. In other words, this form of active immunity is effective in the complete absence of demonstrable type-specific antibodies in the serum of the treated rabbits, and appears to be unrelated to the variety of pneumococcus used for immunization. In attempting to analyze the mechanism underlying this form of effective but non-specific resistance recourse was had to the method previously described (2) by means of which the course of the infection may be followed by observing the intensity and duration of the bacteremia. The course of the bacteremia in 7 of the infected rabbits is given in Table I. The figures presented in the table are typical and representative of the results obtained in the group of animals surviving infection, regardless of the variety of pneumococcus used for immunization, and are equally characteristic of the normal control group. 3 of the animals whose protocols are given, had been previously immunized with a rabbit avirulent strain of Type III, 3 others had been similarly treated with R cells derived from heterologous types, and the remaining rabbit had been immunized with Pneumococcus

Type II. For purposes of comparison the course of the bacteremia during the fatal infection of 2 normal control rabbits is included in the same protocol. The rapidly fatal septicemia in the non-immune animals with progressively increasing and overwhelming numbers of organisms constantly in the blood is in striking contrast to the mild,

TABLE I.

Course of Bacteremia in Immunized Rabbits Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.

(The results given in this protocol are representative of 25 rabbits which survived.)

Rabbit No	1	2	3	4	5	6	7	8	9
Pneumococcus used for immunization	Normal control	Normal control	Type III	Type III	Type III	Type II	R_1 strain	R_1 strain	R_1 strain
Time of culture	No of colonies per unit of blood								
15 min.	∞	∞	49	660	164	261	3200	620	119
2 hrs.	107	∞	1	2	0	12	22	0	37
5 "	393	∞	0	0	3	5	6	1	20
12 "	562	∞	0	6	0	9	12	8	
24 "	∞	D	0	18	0	38	18	3	216
36 "	∞		0	0	4	72	6	10	12
48 "	D		0	0	23	4	5	176	32
72 "			0	0	14	0	5	14	4
4 days			0	0	30	29	3	342	35
5 "			S	S	7	14	15	66	5
6 "					4	6	1	8	6
7 "					7	2	0	6	0
8 "					2	3	6	4	0
9 "					4	0	0	2	0
10 "					1	0	0	0	0
11 "					0	0	0	0	0
					S	S	S	S	S

S indicates survival of animal.
D indicates death of animal.

fluctuating, but progressively decreasing bacteremia which characterizes the course of infection in the immunized rabbits. In many instances, the blood infection in the immune animals may persist for several days, the circulating organisms varying in number from time to time before ultimately disappearing. The non-fatal course of the bacteremia in the treated animals following infection with an S strain

of Type III, highly virulent for rabbits, parallels very closely the curve of the benign bacteremia (2) which occurs in normal rabbits infected with an S culture of Type III, avirulent for this species. The

TABLE II.

Course of Bacteremia in Immunized Rabbits Not Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.

Rabbit No..	10	11	12	13	14	15	16	17	18	19	20	21	22
Pneumococcus used for immunization	Normal controls		Type III	Type III	Type III	Type III	Type III	Type III	Normal controls		R_2	R_1	R_1
Time of culture													
15 min.	∞	∞	29	168	∞	184	330	164	∞	∞	268	4000	564
2 hrs.	∞	3200	9	253	96	15	3	1	3000	∞	4	28	3
5 "	∞	∞	2	406	13	22	3	0	∞	∞	3	5	5
12 "	∞	∞	5	592	24	90	38	0	∞	∞	0	7	9
24 "	∞	∞	6	D	8	15	314	0	∞	D	34	2	2
36 "	D	D	1		4	D		0	D		103	83	5
48 "			251		12		328	23			316	41	4
72 "			133		3		19	14			56	9	24
4 days			316		8		42	30			38	D	33
5 "			93		12		35	7			D		123
6 "			116		216		21	4					D
7 "			D				1200	7					
8 "					72		86	2					
9 "					1500			4					
10 "					D		10	1					
11 "							4	0					
12 "								2					
13 "								1					
14 "							20	6					
15 "							32						
16 "							D						
17 "								3					
18 "													
19 "													
20 "								1					
21 "								D					

D indicates death of animal.

possible significance of the similarity in the course of the bacteremia in both instances and its relation to the mechanism of recovery will be discussed later.

Of the 38 rabbits immunized with pneumococci—25 of which completely recovered from virulent Type III infection—there were 13 animals which died. However, 9 of these may justly be considered as having acquired a considerable though ineffective degree of immunity as a result of the previous immunization. These animals lived 5 to 21 days following infection, whereas the controls all died within 24 to 36 hours. As previously stated, all the experimental animals suffered a massive infection receiving 1 cc. of the virulent Type III culture representing from 1000 to 10,000 minimal lethal doses. If the test had been made less severe by giving smaller infecting doses the number of surviving animals would, in all probability, have been greater.

Not only the duration of life but also the degree of the bacteremia evidenced the presence of resistance in these non-surviving animals. In Table II is given the course of the blood infection in 9 rabbits as estimated by blood cultures taken at frequent intervals. From the table it may be seen that there is an initial sharp reduction in the number of circulating organisms in the resistant animals as contrasted with the controls, and that, although death eventually ensued, the blood infection during life ran a moderately low grade and irregular course, not unlike that in the surviving rabbits. Even in 2 animals (Nos. 13 and 15), which died within 48 hours, and not tabulated as immune, the extent of the bacteremia is markedly less than in the controls. The results indicate that these partially resistant rabbits, although not possessing a solid immunity, were capable of checking the infection, either by inhibiting multiplication of the bacteria or by actually destroying them. Still further evidence that the rabbits, in which death was delayed, possessed some immunity is brought out by the fact that at autopsy, of the 9 examined, 7 suffered from purulent pericarditis and pleuritis, a condition not found in the normals, which died of an overwhelming septicemia. Localization of infection is generally considered as evidence of partial immunity and this has been especially emphasized by Stillman (6) in experimental production of lobar pneumonia. It seems highly probable that the local inflammatory processes were at least partially responsible for the fatal outcome.

In striking contrast to the effective resistance against Type III infection acquired by rabbits immunized with pneumococcus cells is

the absence of protection in other animals immunized with solutions of heterologous pneumococci. The course of the infection in this group shows that they possessed no resistance, at least against a dose as great as 1 cc. of virulent culture. 5 of the 6 rabbits died within 48 hours and 1 lived 3½ days. The bacteremia in these animals was only transiently reduced or entirely unaffected (Table III). Furthermore, none possessed evidence of localization of infection on gross post-mortem examination. It is a striking fact that although these rabbits possessed circulating antiprotein antibodies (anti-P) similar to the rabbits immunized with whole organisms, no increased resistance was

TABLE III.

Course of Bacteremia in Rabbits Immunized with Solutions of Pneumococci and Injected with Rabbit Virulent Strain of Pneumococcus Type III.

Rabbit No	23	24	25	26	27	28	29	30
Material for immunization	Normal control	Pneumococcus nucleoprotein		Normal control	Desoxycholate solution of pneumococcus (R_3)			
15 min.	∞	∞	∞	∞	∞	∞	∞	∞
2 hrs.	∞	∞	∞	∞	175	∞	115	∞
5 "	714	∞	∞	∞	426	∞	217	∞
12 "	∞	∞	∞	∞	∞	∞	382	∞
24 "	∞	D	∞	D	∞	D	∞	∞
36 "	D		D		∞		∞	D
48 "					∞		D	
72 "					∞			
96 "					D			

D indicates death of animal.

apparent. This is strongly suggestive that anti-P antibodies, in themselves, are not significant in this form of active immunity.

DISCUSSION.

The experiments reported in the present paper demonstrate that a considerable degree of increased resistance against virulent Type III pneumococci may be stimulated in rabbits by immunization with homologous or heterologous type-specific S pneumococci or with R forms derived from them. The protection was equally effective regardless of the type of pneumococcal cells used for immunization

(Table IV). The exclusion of a type-specific immune reaction by the use of animals previously treated with heterologous S and R strains, makes it necessary to investigate other factors which might afford an explanation of the active immunity. Singer and Adler (8), in dealing with this problem, concluded that the resistance of immunized rabbits to Type III was dependent upon changes (*Umstimmung*) in reticulo-endothelial cells with which was associated the ability to phagocyte the virulent organisms. Interesting as their experiments were, they were inconclusive in excluding the possible influence of sessile specific antibodies. However, when heterologous S and R

TABLE IV.

Summary of Results in Rabbits Immunized with Homologous or Heterologous Pneumococci and Subsequently Infected with Rabbit Virulent Strain of Type III.

No of rabbits	Immunized with	Type III agglutinins	R agglutinins	No survived	No resistant not surviving	Total No. resistant	Per cent	No. not resistant	Per cent
12	Type III	3+ 9−	+	6	4	10	83	2	17
15	10 Type II 5 Type I	−	+	11	2	13	86	2	14
11	R	−	+	8	2	10	90	1	10
6	Pneumococcus solutions	−	+	0	0	0	0	6	100

pneumococci afford protection, as the present study indicates, type-specific immunological reactions are entirely eliminated. Wright (9) in a recent publication has reported the results of extensive studies on pneumococcus immunity. Employing Type I pneumococci he was able to demonstrate active immunity in rabbits although demonstrable agglutinins were not present in the sera of the immunized animals. However, under the conditions of his experiments, he did not obtain increased resistance to Type I by previous injection of heterologous organisms and concluded that the reaction was type-specific.

Although the experiments reported in this paper have been carried out under conditions which entirely exclude type-specific immunity,

nevertheless, the favorable results obtained under such conditions in no sense minimize the thoroughly established significance of type-specific antibodies in protection against pneumococcus infection. Their effectiveness in sensitizing virulent homologous pneumococci and thereby making phagocytosis possible, has been repeatedly observed.

In seeking for an explanation of the form of non-specific immunity against Type III infection on the basis of circulating antibodies it may be noted that the sera of all the immune animals possessed antiprotein (anti-P) antibodies. Antibodies of this character are reactive with the common pneumococcus nucleoprotein (precipitin) and with all R strains (agglutinin). They are not, however, reactive with encapsulated type-specific organisms nor do they confer passive protection on mice against virulent pneumococci. Consequently it is highly improbable that they are responsible for the disposition of pathogenic Type III organisms. More direct evidence of the ineffectiveness of these antibodies in resistance is brought out by the fact that immunization with pneumococcus solutions (derived from heterologous organisms) fails to afford protection although the sera of rabbits so treated possess anti-P antibodies in high titre. The one factor which all the resistant animals had in common was immunization with formed pneumococcal cells. The nature of the material used as antigen rather than the demonstrable antibody response, therefore, seems to be the significant feature in stimulating this form of active immunity.

Since type-specific antibodies have been excluded, and since the presence of species antibodies (anti-P) does not furnish an adequate explanation for the resistance to Type III, it is necessary to seek further for an understanding of this form of immunity. In a previous paper (2) it was shown that rabbits possess a considerable degree of natural resistance to Pneumococcus Type III, although the strains used for injection were S forms and highly pathogenic for mice. It was also shown that the blood infection resulting from the injection of these *rabbit avirulent* organisms into *normal animals* is characterized by a prolonged course during which the number of circulating bacteria vary from time to time but eventually disappear. In the present paper it is shown that the bacteremia occurring in an *immune rabbit* injected with a *rabbit virulent* strain runs a strikingly similar course.

Consequently it seems possible that the explanation of acquired resistance, in this instance, is due not to antibodies which have been elicited, but to an increased effectiveness of the mechanism of natural resistance. Wright (9) in his recent publication has offered a similar explanation for the active immunity which he obtained and he considered the difference between normal and immune rabbits to be quantitative and not qualitative. However, the emphasis which he places upon specificity makes it necessary to assume that normal rabbits possess type-specific protective susbtances, a conception which Sia (10) previously suggested and more recently (11) has further emphasized. The experiments reported in this paper reveal the fact that enhanced resistance to Type III pneumococci may be stimulated in rabbits by previous injections of any *R* or *S* pneumococcus cells, and, according to the explanation advanced, is due to an exaltation of the same factors which endow normal rabbits with natural resistance to Type III infection. Further experiments tending to substantiate this view will be subsequently reported. If this hypothesis proves correct, these experiments also tend to show that, whereas intact cells stimulate the processes of natural resistance, the same material in solution is ineffective, although a similar antibody response (anti-P) is elicited in both instances.

SUMMARY.

1. Immunization of rabbits with Type III pneumococci is effective in producing active immunity against infection with a virulent strain of the homologous organism.

2. Immunization of rabbits with Type I or II pneumococci, and with R forms derived from any of the fixed types, is equally effective in producing active immunity against Type III infection.

3. Immunization of rabbits with nucleoprotein or with desoxycholate solutions of heterologous pneumococci, under the experimental conditions described, appears to be ineffective in producing active immunity against Type III infection.

CONCLUSIONS.

Increased resistance against virulent Type III pneumococci may be stimulated in rabbits by repeated injections of heat-killed cultures

of homologous or heterologous pneumococci. This form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of the pneumococcus used for immunization, is considered dependent upon an exaltation of the same factors which afford normal rabbits natural resistance to Type III pneumococcus.

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[Reprinted from The Journal of Clinical Investigation, August 20, 1927, Vol. iv, No. 3, pp. 307–315.]

STUDIES ON HUMAN CAPILLARIES.

IV. Observations on the Nature of the Capillary Pulse in Aortic Insufficiency.

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(Received for publication, April 1, 1927.)

The first description of the capillary pulse was given in 1868 by Quincke (1) who observed it in a number of different conditions but stated that it was most manifest in cases of aortic insufficiency. He thought that the mechanism involved was a true pulsation in the capillaries, that is to say, a pulsatile movement in the capillaries resulting from cardiac systole. The phenomenon has come to be commonly recognized in clinical medicine and until recently it was assumed that it was due to actual pulsation in the capillaries. Since it has been shown, however, that the capillaries can be viewed microscopically the mechanism involved has been studied by several investigators but no agreement has been reached as to its nature. In conditions in which a capillary pulse is present there is apt to be a pulsatile movement of the whole part examined due to the heart beat, and as this takes place at the precise moment when the changes for which one is looking would take place, the greatest care must be observed in carrying out the observations so that in consequence of the motion involved the capillaries do not slip out of focus.

Before the advent of capillary microscopy Herz (2) expressed doubt as to whether pulsation took place in the capillaries and thought rather that it did so in the arterioles. As the result of his microscopic observations of the skin capillaries Jürgensen (3) differentiated two groups of cases; one with a true capillary pulse in which there was an actual pulsation of the stream within the capillaries and the other with a pseudo-capillary pulse in which there was no pulsation of the stream. In the latter the impression of pulsation was given to the capillaries

of the superficial layers by pulsation transmitted to them by the pulse of deeper lying vessels such as the digital arteries and arterioles. The former he thought was most often present in aortic insufficiency and the latter was seen in many cases of arteriosclerosis. Freedlander and Lenhart (4) as the result of their studies arrived at similar conclusions. Weiss and Dieter (5) described a pulsatile flow in the capillaries in aortic disease while Secher (6), Fischl (7) and Hisinger-Jagerskiöld (8) came to the conclusion that the phenomenon was not due to pulsation of the blood stream within the capillaries. Secher saw pulsation in the capillaries in a few cases of his series but Hisinger-Jagerskiöld only observed it in one of his. Boas (9) studied the capillaries of the nail fold in cases of aortic insufficiency and hypertension and came to the conclusion that the capillary pulse was not a manifestation of pulsation of the capillaries but was due to an exaggerated pulsation of the arterioles and possibly of the venules of the sub-papillary plexus. Sumbal (10) investigated the capillaries of the lip in cases of aortic regurgitation and stated that pulsation could be seen in every case in the capillaries in which the flow was not too rapid. As the result of Sumbal's work Boas (11) reinvestigated the subject and studied the capillaries in various situations. He was able to demonstrate actual pulsation in the capillaries in some patients with aortic insufficiency but he did not think it was present with sufficient constancy or intensity to warrant adopting it as the explanation of clinical capillary pulsation. Lewis (12) has more recently carried out an extensive study of the question. He regarded macroscopic capillary pulsation as a physiological phenomenon which can readily be induced in normal individuals by heating the part examined and thus causing dilatation of the arterioles. He stated that anything which induces arteriolar dilatation will bring about pulsatile flow in the capillaries. He never failed to observe pulsation in the capillaries of any area in which pulsation of color was visible to the naked eye and in which the blood flow in a reasonable number of capillaries could be detected. He thinks that every case, regardless of etiology, in which clinical capillary pulsation, that is to say a change of color, is evident, shows pulsation in the capillaries due to dilatation of the arterioles. When macroscopic pulsation is observable the pulse passes from the arterioles through the capillaries

to the venules and the pulsation of these plays a greater or lesser part in the color changes observed according to the situation examined. Heimberger (13) found in normal individuals that mechanical irritation of the arterial limb of the capillary or the local application of drugs which dilate them causes pulsation to appear. Pulsation also resulted from light pressure on the venous limb or from heat. He concluded that in normal individuals the pulse wave was most often lost in the arterial limb of the capillaries and could be seen throughout the whole capillary only when the tone of the arterial limb was reduced. To summarize these statements one may say that two views are held as to the main cause of "capillary pulsation" observed in cases of aortic insufficiency: (*a*) that it is due to pulsation of the blood stream within the capillaries and (*b*) that it is due to an exaggerated pulsation in the larger vessels which lie deeper.

The results to be reported are based on the study of twelve cases of aortic insufficiency in which there was well marked macroscopic capillary pulsation. Seven of these were of rheumatic origin, four of syphilitic and one was due to subacute bacterial endocarditis. None of the cases showed marked signs of cardiac decompensation. Cinematographic records of the capillaries at the nail fold and electrocardiograms (Lead II) were made simultaneously. Synchronous points were recorded on the cinematographic film and electrocardiograms by means of signals which were operated by a common switch. By this means it was possible to calculate the precise phase of the particular cardiac cycle to which each cinematographic photograph corresponded. The details of the technique of making the cinematographic exposures and the methods employed in their study have been previously described (14, 15).

OBSERVATIONS

Changes in calibre. The average size of both the arterial and venous limbs was similar to that seen in normal subjects. Variations in the diameter took place continuously. The extent of these changes varied from capillary to capillary in the same subject and also from subject to subject. The magnitude of the variations in calibre was greater in the arterial limb in many instances than had been observed

in normal individuals (fig. 1). The venous limb as a rule showed changes in diameter which corresponded more closely with the normal.

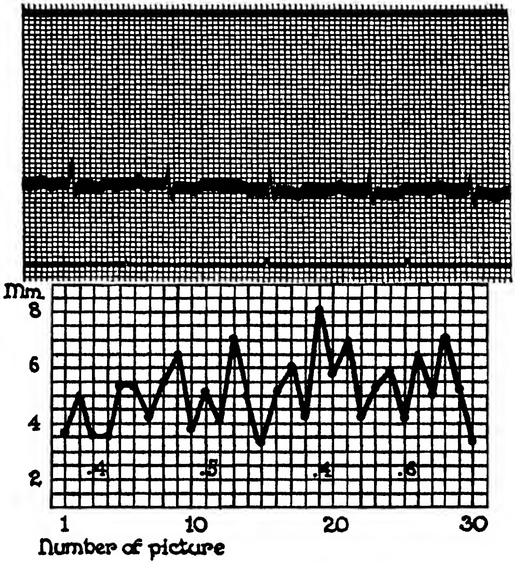


FIG. 1. AN ELECTROCARDIOGRAM (LEAD II) IS SHOWN FROM A CASE OF AORTIC INSUFFICIENCY IN WHICH MACROSCOPIC CAPILLARY PULSATION WAS VISIBLE

The electrocardiogram was taken at the same time as cinematographic records were made of the capillaries at the nail fold. Synchronous points of the electrocardiograms and of the cinematographic exposures were identified by means of two signals in a single circuit one recording on the electrocardiograph film and the other on the cinematograph film. Underneath the electrocardiogram are shown the changes which took place in the diameter of the arterial limb of a capillary during identical cycles. Each measurement is actually synchronous with the point in the electrocardiogram immediately above it without allowance for the probable transmission time.

The ordinate represents the diameter of the arterial limb in millimeters at a point 2 cm. from the tip of the capillary loop after magnification of the capillary 350 times. The number of cinematographic exposures was 10 per second. The decimal numbers indicate the time in seconds after the onset of ventricular systole (taken as the beginning of the R-wave of the electrocardiogram) at which the maximum diameter of the arterial limb was present in each cardiac cycle.

Evidences of pulsation due to the heart beat. The chief interest in this investigation consists in the study of the relation of the changes

in the capillaries to the pulse beat, to see whether the capillaries show rhythmic changes in diameter such as would take place if a pulsatory flow of blood were present in the capillaries themselves. Changes took place constantly in both limbs of the capillary loops but as a rule were more marked in the arterial limb. These were, however, totally irregular and showed no rhythmicity such as one would expect if they were due to pulsation of the blood stream in the capillary (fig. 1). If the mechanism involved were of a pulsatory nature there ought to be a constant time relation between the onset of ventricular systole—taken as the beginning of the R-wave of the electrocardio-

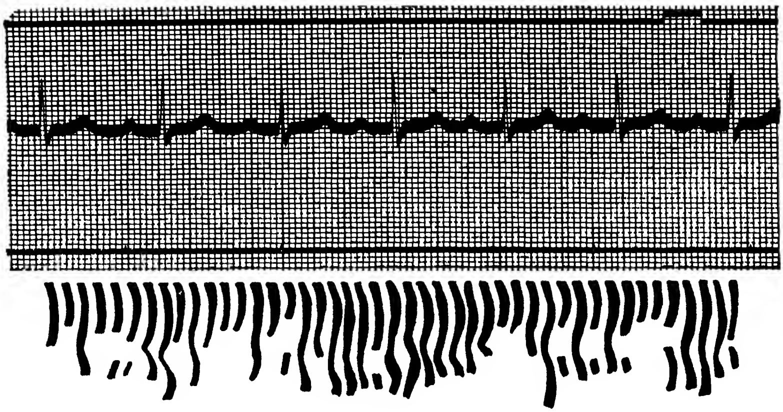
TABLE 1

The time in seconds after the onset of ventricular systole at which the maximum diameters of the limbs of the capillary loop occurred

Cycle	Arterial	Venous
	seconds	*seconds*
I	0 41	0 71
II	0 57	0.27
III	0.71	0 61
IV	0 31	0.11
V	0.30	0 40
VI	0.27	0 67
VII	0.29	0 59
VIII	0.63	0 53
IX	0.67	0 67
X	0.41	0 51
XI	0.55	0 55
XII	0.49	0 09
XIII	0.33	0 63

gram—and the occurrence of the maximum diameter of the arterial and possibly also of the venous limb of the capillary. This time was calculated in a large number of cardiac cycles but was not constant so that no evidence of pulsation in the capillaries is afforded by this means (table 1). In a series of cycles which have been measured (table 1) the time varied in the case of the arterial limb from 0.27 to 0.71 second and in the venous limb from 0.09 to 0.71 second. In one subject the flow of blood in the capillary was frequently interrupted so that the length of the arterial limb in which blood was visible varied constantly. On superficial examination it looked as if

pulsation were present. The length of the arterial limb in a large series of consecutive photographs was traced and studied in conjunction with the electrocardiogram. If the changes were due to pulsation there ought to have been a rhythmic filling and emptying of the arterial limb. Most blood ought to have been visible in it at a constant



0.71 0.65 0.59 0.73 0.17 0.25

FIG. 2. AN ELECTROCARDIOGRAM (LEAD II) IS SHOWN FROM A CASE OF AORTIC INSUFFICIENCY IN WHICH MACROSCOPIC CAPILLARY PULSATION WAS VISIBLE

Simultaneous cinematographic records of the capillaries at the nail fold were made. Synchronous points were ascertained by the technique described in figure 1. Underneath the electrocardiogram are drawings of the visible portion of the arterial limb of the same capillary in all the exposures taken when this electrocardiogram was made. Each drawing is actually synchronous with the point in the electrocardiogram immediately above it without allowance for the probable transmission time.

The number of cinematographic exposures was 10 per second. The drawings were made from tracings which magnified the size of the capillary 350 times. The decimal numbers indicate the time in seconds after the onset of ventricular systole in each cycle at which blood was visible in the greatest length of the arterial limb.

time after the onset of ventricular systole. This time should correspond with that at which the heart beat produced its maximum effect in the periphery. The changes seen, however, did not fulfill these expectations (fig. 2, table 2). The time in seconds after the onset of ventricular systole at which the greatest length of the arterial limb

was visible differed in the cycles illustrated in figure 2 from 0.17 to 0.73 second and in those shown in table 2 from 0.01 to 0.83 second. In some cycles the arterial limb was longest at the time one would expect cardiac systole to pump more blood into the capillaries, but in others this occurred at a later period and in a few just before the onset of the next ventricular contraction. In many instances marked irregularity was seen during an individual cycle. The type of variation is well illustrated in figure 2. In cycle I the length of the arterial limb was short at the beginning. It became longer, shortened again, and was longest immediately before the next ventricular complex.

TABLE 2

The time in seconds after the onset of the corresponding ventricular systole (1) at which the maximum diameter of the arterial and venous limbs occurred, and (2) at which the arterial limb exhibited its greatest length

Cycle	Arterial	Venous	Longest arterial limb
	seconds	*seconds*	*seconds*
I	0 60	0 10	0 80
II	0 51	0 21	0 01
III	0 43	0 33	0 73
IV	0 47	0 77	0 47
V	0 29	0 29	0 59
VI	0 31	0 21	0 01
VII	0 65	0 35	0 35
VIII	0 57	0 57	0 07
IX	0 27	0 27	0 67
X	0 69	0 19	0 29
XI	0 83	0 43	0 83
XII	0 21	0 11	0 21

In cycle II the limb was also very variable, being long at the beginning and also towards the end. The amount of blood visible in the arterial limb in cycle III was greater than in the two previous cycles and remained approximately the same throughout. Cycle IV showed a progressive decrease throughout, while in cycle V the total length remained approximately the same except once near the beginning when it became longer; at other times small gaps in the stream took place. In cycle VI the limb presented a gap and then became much shorter but increased considerably at the middle and end, the length remaining about the same throughout these periods.

Blood flow. The capillaries at the nail fold were studied by inspection over prolonged periods of time. In this condition the difficulty of keeping the capillaries under observation which has been described by previous authors is especially great as the finger usually moves with every pulse beat. By careful fixation of the finger the extent of this movement can be reduced to a minimum but even then it is necessary to focus very carefully all the time in order to see the flow clearly. Variations in flow took place continuously in the various capillaries. In the greater number these changes bore no relation to the pulse beat and resembled those seen in normal individuals. In a few, changes in the rate of flow were present for a short time which suggested pulsation but it was impossible to be sure that this was the mechanism involved.

SUMMARY AND DISCUSSION

These studies show that the diameter of the capillaries in cases of aortic insufficiency was constantly changing. The various capillaries differed in this respect and no two subjects were alike. The blood flow also was very variable. The type of change observed resembled that seen in normal individuals. In several instances the magnitude of the variations in the arterial limb was greater than was seen in normal subjects and in patients with heart disease without cardiac decompensation. The changes in the venous limb resembled those in normal subjects. The mechanism of the production of these changes is uncertain. The possible factors have been discussed in a previous paper (14). No evidence was found o pulsation in the capillaries of the nail fold although macroscopic pulsation was present in the vessels under the nail and in those behind the nail fold. The site chosen for observation is not the most suitable for displaying the phenomenon but is the only one at present available with our technique. It is possible that pulsation might be seen in the capillaries in a more favorable situation.

CONCLUSIONS

1. The calibre of the arterial and venous limbs of the capillaries at the nail fold has been investigated by means of cinematography in 12 cases of aortic insufficiency in which macroscopic capillary

pulsation was present. Simultaneous electrocardiograms were made so that the relation of the capillary changes to the heart beat could be studied.

2. Variations in the diameter of the arterial and venous limbs took place continuously. The changes in the arterial limb in some subjects were more marked than those seen in normal individuals but the changes in the venous limb as a rule were not so marked as in the arterial and resembled those seen in the normal.

3. No evidence was present to indicate that pulsation due to the heart beat was present in the capillaries examined.

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[Reprinted from The Journal of Clinical Investigation, August 20, 1927, Vol. iv, No. 3, pp. 317–322.]

STUDIES ON HUMAN CAPILLARIES.

V. Observations in Cases of Heart Disease with Regular Rhythm.

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(Received for publication, April 1, 1927.)

In previous studies of the capillary circulation it was found that changes in the calibre of the capillaries took place continuously, both in health when the rhythm of the heart is regular, and in auricular fibrillation. The extent of these changes appeared to depend on the state of circulatory compensation and was independent of the rate or regularity of the pulse. Similar variations were observed in advanced decompensation both in cases of regular and of irregular rhythm. The present investigation was undertaken to study the changes which take place in heart disease with regular rhythm from the early to the late stages of the disease. Weiss (1) found in his studies of the capillaries in living subjects that cases of mitral insufficiency without cardiac decompensation showed a normal picture, while in the early stages of mitral stenosis a distinct slowing of the stream was observed. Cases of cardiac decompensation, however, showed marked dilatation of the venous limb and the blood stream was slow and had a granular appearance. These findings were in the main confirmed by Schur (2), Jürgensen (3), Neumann (4), Secher (5), Freedlander and Lenhart (6). Secher, however, described a normal capillary picture in this condition in spite of symptoms of cardiac insufficiency. Hisinger-Jagerskiöld (7) studied a large number of cases of valvular and myocardial heart disease with regular rhythm. He stated that cases with full compensation or with only slight decompensation did not differ from normal subjects but those with clinical signs of decompensation as a rule showed the changes described by Weiss in this condition. He

further described a group of cases which showed congestion of internal organs but otherwise no clinical signs of cardiac decompensation. In these the size of the loops was normal and the picture closely resembled that which the author associated with anemia.

The results to be described are based on the study of eight cases of heart disease with regular rhythm, seven of which were of rheumatic origin while the other was one of chronic myocarditis. These cases varied in their severity from a degree in which a heart lesion had just developed to one in which cardiac decompensation was marked. Cinematographic observations of the capillaries at the nail fold and simultaneous electrocardiograms were made. Synchronous points were recorded both on the photographic and the electrocardiographic films. The details of taking the cinematographic exposures and the method employed in their study have been reported in previous papers of this series (8, 9). The technique used in recording synchronous points on the cinematographic and electrocardiographic films has also been described in a previous paper (10).

The size of the loops. There was marked variation in the size of the loops in the same subject and differences were also seen between individual subjects. The state of compensation markedly affected the size of the loops, especially the venous limb. The average size of the arterial limb in early cases was from 0.014 to 0.015 mm., while that of the venous was from 0.015 to 0.016 mm. In advanced cases, the arterial limb varied from 0.015 to 0.017 mm. and the venous from 0.017 to 0.018 mm. The early cases thus presented a picture such as had been observed in normal individuals while the advanced cases resembled that seen in auricular fibrillation with clinical signs of decompensation. The values given are those of most of the loops measured, and although larger and smaller loops were studied the changes observed were similar in all.

Variations in calibre. Variations in the diameter of the loops of about equal extent in both the arterial and venous limbs took place in the same subject from moment to moment. The magnitude of these changes varied in the loops of the same subject and also in different subjects. The alterations were comparatively small, however, compared to the total breadth of the limb which remained approximately the same. Their magnitude was definitely influenced by the state of

cardiac compensation. In the early stages the variations were less marked than in the later stages (figs. 1 and 2).

Evidence of independent contractility of the capillaries. Curves which were prepared in a manner similar to those described in previous papers in this series have been studied to see whether there was evi-

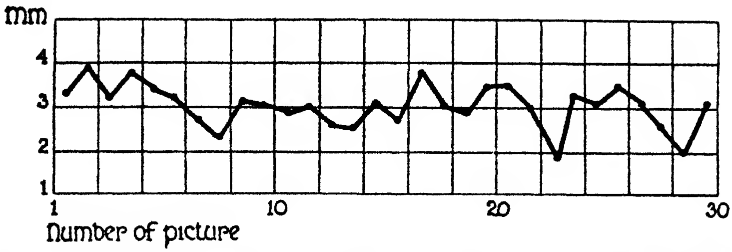

FIG. 1. THE CHANGES ARE SHOWN WHICH TAKE PLACE IN THE DIAMETER OF THE ARTERIAL LIMB OF A CAPILLARY IN AN EARLY CASE OF MITRAL STENOSIS. × 350

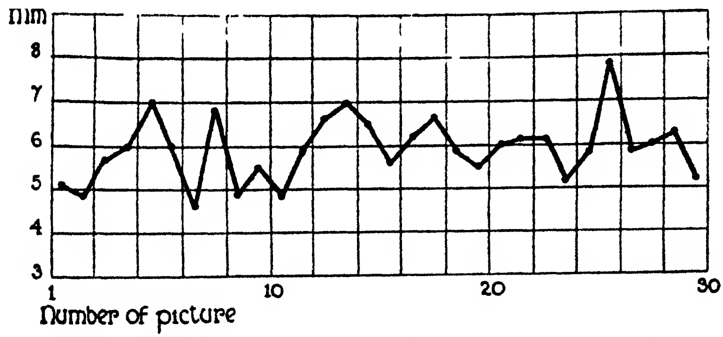

FIG. 2. THE CHANGES ARE SHOWN WHICH TAKE PLACE IN THE DIAMETER OF THE ARTERIAL LIMB OF A CAPILLARY IN A CASE OF CHRONIC MYOCARDITIS WITH ADVANCED HEART FAILURE. × 350

dence of a peristaltic wave of the capillaries or of local rhythmical contractions in them. The changes in diameter took place irregularly and no rhythmicity was seen so that neither of these mechanisms appears to be the cause of their production.

Evidence of pulsation due to the heart beat. The method of recording simultaneously the capillary changes and the electrocardiogram en-

abled one to study whether any of those observed depend on pulsation on the capillary wall, due to an effect on the blood stream incident to cardiac systole. The duration of each cardiac systole could be accurately measured. If the changes were of a pulsatile nature the maximum diameter of the limbs of the capillary loops ought to occur at a constant time after the onset of each ventricular systole. All the curves have been analyzed from this point of view but the time at which the maximum diameter occurred has no relation to the onset of cardiac systole. It appears, therefore, that cardiac pulsation does not account for the changes observed.

Blood flow. The blood flow was studied by inspection over prolonged periods of time. The rate of flow was continually changing in the same capillary; different capillaries in the same subject also varied in this respect. In early cases the stream was usually rapid and resembled in every respect that seen in normal subjects. In the more advanced cases the rate of flow was slower while in those with advanced heart failure the stream was very slow and in many cases stasis was present for considerable periods of time. Marked sudden variations were often seen and gaps in the corpuscular stream were frequently present. The general appearance was similar in every respect to that seen in cases of auricular fibrillation with advanced heart failure.

Digitalis. The more advanced patients were studied both before and after thorough digitalization. Only one case derived definite clinical benefit. In this case the extent of the variations in the diameter of the limbs of the capillaries was reduced while the blood flow improved in a corresponding manner. In the other cases which were uninfluenced by digitalis there was no change observed in the capillary circulation.

DISCUSSION

The variations which took place in the calibre of the capillaries in heart disease with normal rhythm were of a similar nature to those which had been observed in normal subjects. Those cases which showed no clinical signs of heart failure did not differ in any respect from the normal, while advanced cases showed changes of the same nature but of greater magnitude. They corresponded closely to the changes which were seen in cases of auricular fibrillation with a com-

parable degree of decompensation. The extent of these variations bore no relation to the pulse rate but seemed to correspond to the state of efficiency of the circulation as judged by the clinical condition of the patient.

The blood flow in the capillaries showed a similar correspondence to the state of cardiac compensation.

No evidence was found of independent contractility of the capillaries, nor were the changes due to a pulsatile motion conveyed to the blood stream by the heart beat. Their cause is in doubt. Their nature is similar to that observed in normal cases. The possible factors involved have been discussed in an earlier paper (9). The differences which exist appear to depend on the power of the heart itself to maintain an efficient circulation.

CONCLUSION

1. The calibre of the arterial and venous limbs of the capillaries at the nail fold has been studied by means of cinematography in eight cases of heart disease with normal rhythm.
2. Changes of equal magnitude in the diameter of the arterial and venous limbs of the capillary loop take place from moment to moment. The extent of these changes varies in different capillaries in the same subject; differences are also seen between individual subjects.
3. The magnitude of the variations depends on the state of cardiac compensation and has no relation to the rate of the pulse.
4. The changes do not appear to be due to a peristaltic wave of contraction, a local rhythmical contraction of the capillaries nor to the action on the capillary wall of a pulsatory motion of the blood stream caused by the heart beat. The cause of their production is uncertain.
5. The blood flow in cases without cardiac decompensation is similar to that seen in normal subjects while in cases with cardiac decompensation the flow is slow and irregular such as was observed in cases of auricular fibrillation with a comparable degree of decompensation.

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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, June, 1927, Vol. lxxiii, No. 2, pp. 695–723.]

DETERMINATION OF UREA BY GASOMETRIC MEASUREMENT OF THE CARBON DIOXIDE FORMED BY THE ACTION OF UREASE.

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(Received for publication, April 30, 1927.)

Urea is changed by the action of urease into ammonium carbonate: $CO(NH_2)_2 + 2\ H_2O = (NH_4)_2CO_3$.[1] The ammonia has been more commonly determined as a measure of the urea. However, as shown by Partos (3) and by Mirkin (2), one can also obtain an exact estimate by determining the CO_2 of the ammonium carbonate. The manometric blood gas apparatus (7, 4) is particularly adapted to this determination, because of the wide range over which it yields accurate results. In practice the gasometric CO_2 urea determination has proved to have several advantages over the ammonia estimation. The gasometric method dispenses with the apparatus required for aeration or distillation of the ammonia, and with the necessity for exact standard solutions for titration, or for colorimetric comparison in Nesslerization. The result is a diminution in the sources and likelihood of error and, at least in the case of urine analyses, a gain in rapidity.

Preformed carbonic acid and bicarbonate exist in both blood and urine. This CO_2 is removed by acidifying and agitating the blood or urine before the urease is added. In analysis of urine the CO_2 is removed in a volumetric flask by acidifying with phosphoric acid and whirling the fluid about the walls of the flask. Sodium hydroxide is then added in amount to form the optimum phosphate buffer mixture for the action of urease. The urease is then added, with sufficient

[1] For experiments and literature concerning the mode of action of urease and the optimum conditions for its action see Van Slyke and Cullen (5) and Van Slyke and Zacharias (9).

water to dilute the urine sample either 10- or 20-fold. After the urease has acted 20 minutes samples of the solution may be drawn for CO_2 determinations. The latter, by the technique described below, can be easily performed in series at the rate of a determination every 4 minutes. This procedure has been in continual use for routine urine analyses in this laboratory for about a year.

A similar technique is applicable to the Folin-Wu filtrate of blood, and has proven satisfactory in routine hospital analyses. In this case it is convenient to let the urease and aerated filtrate react inside the gas apparatus, a procedure which prolongs the time for a determination only by the 1 minute required for the enzyme to act.

The removal of the blood proteins is not a necessary preliminary to the analysis. The determination can be performed directly on either whole blood or plasma. The only drawback is that the determination requires 15 minutes, compared with the 5 for the Folin-Wu filtrate. In the direct analysis of whole blood or plasma the preliminary removal of CO_2 is most conveniently performed in the gas apparatus itself. The blood sample is introduced, acidified with lactic acid, and freed of CO_2 by extraction *in vacuo*. Na_3PO_4 is added in such amount as to form an optimum phosphate buffer mixture; *viz.*, with $Na_2HPO_4:NaH_2PO_4 = 1:1$ (9). The urease solution is added to the blood solution so prepared, and, with the enzyme we have employed, it has been necessary to wait only 1 minute for it to act, even with uremic blood. An excess of lactic acid is finally added, and the CO_2 is extracted and measured by observation of the gas pressure before and after absorption with sodium hydroxide.

In accuracy there is no difference between determination of the CO_2 and that of the NH_3 formed by the action of urease, if samples of size best suited for the measurement of each are taken. In urine analyses this is the case. In blood analyses the results obtainable with 1 cc. samples by the gasometric method are about equal in accuracy (1 per cent of the amount measured) to those obtainable with 5 cc. by the ammonia titration method, and considerably more accurate than those usually obtainable by titration with the 2 cc. samples commonly used. Gasometrically one can obtain with a little practice very satisfactory accuracy, by the micro technique described below, with 0.2 cc. of blood taken from an ear puncture.

Determination of Urea in Urine.

Reagents.

Urease Solution.—We have used a 10 per cent water solution of Squibb's urease, which is prepared from jack beans by Van Slyke and Cullen's acetone precipitation method (6). We have always found this of full activity and free of CO_2. Any other CO_2-free urease preparation may presumably be used, if the activity or the time required by it to decompose completely the maximum amount of urea encountered in human urine (3 per cent) is determined. The urease solution in 50 per cent glycerol prescribed for blood analysis may also be used for urine.

1 N Lactic Acid (Approximate).—Made with sufficient accuracy by diluting 1 volume of commercial lactic acid of 1.20 specific gravity to 10 volumes with water.

2 M H_3PO_4.—Dilute 13.2 cc. of syrupy H_3PO_4, of specific gravity 1.725, to 100 cc.

Saturated Carbonate-Free NaOH Solution.—Sodium hydroxide is dissolved in an equal weight of water and permitted to stand in a closed vessel until the carbonate has settled. The concentration of NaOH is approximately 18 N.

2 M Carbonate-Free NaOH.—Dilute 11.0 cc. of the saturated sodium hydroxide to 100 cc. At once draw the solution up into a soda-lime tube (Fig. 1) protected at the top by soda-lime from atmospheric CO_2.

Brom-Thymol Blue, 0.4 Per Cent Solution.—100 mg. of the powdered dye are ground in a mortar with 3.0 cc. of 0.05 N NaOH, and then diluted to 25 cc. with water.

Caprylic-Ethyl Alcohol.—1 volume of caprylic alcohol is mixed with 4 volumes of 95 per cent ethyl alcohol. This mixture is used to prevent foaming.

Test of 2 M H_3PO_4 and 2 N NaOH.

A portion of 2 M H_3PO_4 may be titrated with the 2 N NaOH, using phenolphthalein as indicator, and titrating to a full red. Each cc. of phosphoric acid neutralizes 2 cc. of sodium hydroxide. A variation of 10 per cent may be permitted; *i.e.*, 1 cc. of H_3PO_4 may neutralize from 1.80 to 2.20 of NaOH.

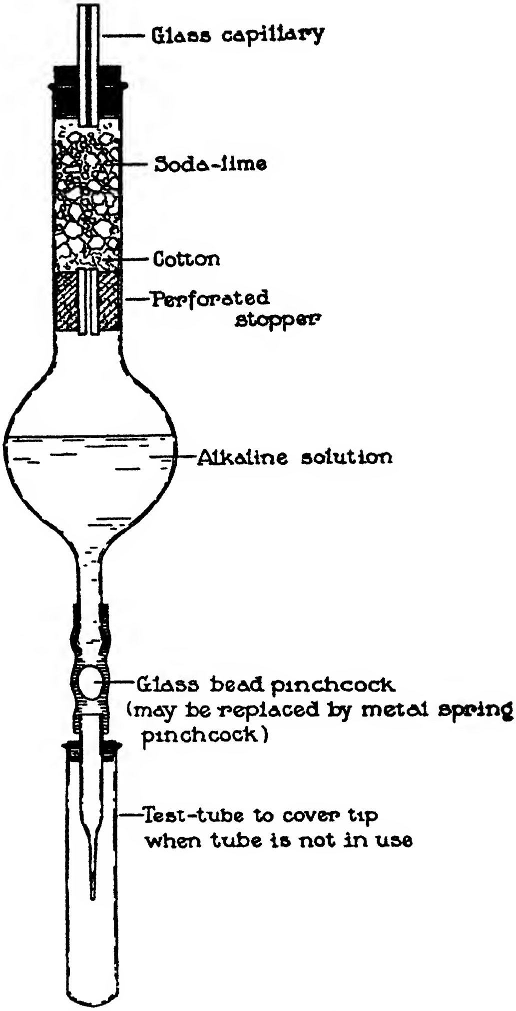

FIG. 1. Soda-lime tube of about 100 cc. capacity for holding CO_2-free alkali or alkaline phosphate The tip is drawn out quite fine, so that it delivers 35 to 40 drops per cc., the exact number being determined by trial with the solution used and marked on the tube. The form of cock shown, made by a glass bead in a rubber tube, is convenient for delivery of drops by count. By pinching the tube about the bead delivery of the fluid is carried at the desired rate. An ordinary metal pinch-cock may also be used.

The solutions may also be tested as follows: To 20 cc. of water add 1 cc. of each solution. Mix and pour half into each of two test-tubes. To one add methyl orange indicator, to the other add sodium alizarin sulfonate. Both should become yellow, indicating that the solution is alkaline to methyl orange, acid to alizarin red, and is therefore at a pH between 4 and 5.

The 2 N NaOH should be titrated, or tested as above, when it is made up, and once a week thereafter. If the solution stands for some weeks or months in the soda-lime it is likely to increase in concentration by more than the permitted 10 per cent, as the result either of losing water by slow distillation up to the soda-lime, or of solution of more alkali from the glass. An intact coat of paraffin on the tube should prevent reaction with the glass. It is advisable even with a paraffin-coated tube, however, to test alkali that has stood in it for some time.

Procedure for Urea Determination in Urine.

Removal of Performed CO_2.– Place in a 20 cc. measuring flask 2 cc. of urine of specific gravity below 1.030, or 1 cc. of more concentrated urine. Add 0.25 cc. of 2 M H_3PO_4, which may be measured conveniently and with sufficient accuracy by drops delivered from the fine glass tip of a dropping pipette, and 3 drops of brom-thymol blue solution. Whirl the urine about the walls of the flask for 1 minute to permit CO_2 to escape. Insert a tube into the flask and draw air through it to sweep out the CO_2 gas. Then whirl the urine again for a minute to permit escape of the last traces of CO_2. (This second treatment is necessary only for urines with such high bicarbonate content that they are alkaline to litmus, but it is probably safer and as little trouble to carry it through as a routine procedure on all.)

Digestion of Urine with Urease.—Dilute to about 18 cc. Add 0.35 cc. of 2 N NaOH, measured by drops from the tube (Fig. 1) in which it is stored. (The first drops from the tube are discarded, as the solution at the capillary tip is likely to have taken up CO_2 from the air.) Then add 1 cc. of urease solution from a pipette which dips below the surface of the diluted urine. The heavy urease solution sinks to the bottom of the flask, and does not decompose the urea at the top until

after the solutions are mixed. The solution is now diluted up to 19.9 cc., and the flask is closed with a 1-hole rubber stopper, the hole of which is filled by a vaselined glass rod (Fig. 2). The contents of the flask are mixed, and allowed to stand 20 or more minutes for the enzyme to act. The mixed solution should take on a distinctly green color, and may quickly become blue from formation of ammonium carbonate. If it does not change from yellow to at least green (pH

FIG 2. Volumetric flask for urine analysis The lower end of the glass rod and the perforation through the stopper are vaselined before each analysis.

about 6.5), add another drop of 2 N alkali through the hole in the stopper.

Addition of Excess Alkali to Digested Urine to Prevent CO_2 Loss.—At the end of the 20 minutes or longer period the glass rod is withdrawn from the stopper, and, after the first 3 drops of alkali have been discarded, the capillary tip of the tube (Fig. 1) delivering 18 N NaOH is inserted through the hole. 0.2 cc. of the concentrated alkali is added measured by (about 5) drops. It is advisable to vaseline the delivery tip, so that the alkali will fall cleanly from the end

and not creep back up the sides. The 0.2 cc. of concentrated alkali increases the volume of the solution by only 0.1 cc. The stopper is again closed with the glass rod, and the flask is inverted 2 or 3 times to mix the alkali with the solution, and to absorb any CO_2 that may have escaped into the gas space beneath the stopper. The 0.2 cc. of concentrated NaOH makes the solution so alkaline that there is no danger of loss of CO_2 when the flask is subsequently opened for withdrawal of samples. To prevent absorption of atmospheric CO_2, the flask is kept closed, except when samples are pipetted out for analysis.

Extraction and Measurement of CO_2 Formed from Urine Urea.—Place a drop of the caprylic-ethyl alcohol mixture in the capillary and 1 cc. of water in the cup of the 50 cc. blood gas apparatus of Van Slyke and Neill (4, 7). Run 2 cc. of the digested urine solution under the water. Admit the urine solution into the chamber of the apparatus followed by the 1 cc. of water. Then add 0.5 cc. of 1 N lactic acid. Evacuate the chamber and shake $1\frac{1}{2}$ minutes. Reduce the gas volume to 2 cc. as described for CO_2 determinations in blood on p. 533 of Van Slyke and Neill's paper (7). Read pressure p_1 on manometer, and eject the solution from the apparatus.

Run a control with reagents alone, without any urine. The manometer reading for the control is p_0.

The pressure due to CO_2 from urea is $P_{urea} = p_1 - p_0$.

One control serves for an entire series of urea determinations. If the temperature changes while the series is being analyzed, correct p_0 by adding or subtracting 1.3 mm. for each degree rise or fall of temperature centigrade. This serves to correct for the change in the vapor tension of the water in the apparatus, and in the pressure exerted by the air extracted from the solution, if the temperature does not vary more than 3°. If it does, repeat the analysis of the control solution.

Remarks on the Urine Analysis.

In running a series of analyses it is not necessary to wash the gas apparatus between determinations. Since the CO_2 is not reabsorbed with alkali in the apparatus, the ejection of the gas and the acidified solution after each analysis removes the CO_2 with sufficient complete-

ness. One may consequently proceed with the analyses at the rate of one every 4 minutes, and have sufficient leisure to calculate each result during the extraction in the next analysis.

The 1 cc. of water and the 0.5 cc. of lactic acid solution added to the urine solution in the apparatus should be measured to within 0.05 cc. The dissolved air in the 3.5 cc. of solution is extracted and measured with the CO_2, the correction for the air content being included in the blank. 0.1 cc. of water saturated with air at room temperature yields sufficient air to exert at 2 cc. volume about 1 mm. pressure in the apparatus, and it is desirable to keep the error within this limit. Hence the 3.5 cc. volume of water solution should be measured to within 0.05 cc. The 1 cc. of water first placed in the cup should be measured from a 1 cc. pipette, rather than by the mark on the cup itself.

Gasometric Urea Determination in Folin-Wu Blood Filtrate.

The determination may be made, as in the urine analysis described above, by measuring the total gas (air + CO_2) after the action of urease, and subtracting the dissolved air obtained in a blank analysis. This procedure (A) is the one of choice for speed and convenience when a number of blood urea determinations are required; each determination is simplified to one extraction and one reading of the manometer, and, as the CO_2 is not reabsorbed with alkali, the apparatus need not be washed between determinations. For the blank analysis, 0.9 per cent NaCl solution is used, because it dissolves the same amount of gas as the Folin-Wu filtrate; *viz.*, 94.5 per cent as much as does water.

When only one or two analyses are to be made, it is simpler to obviate the blank, and perform in the gas apparatus the extraction of preformed CO_2, as well as the subsequent analysis. Accordingly both procedures will be described.

Reagents for Blood Filtrate Analysis.

0.9 per cent NaCl solution, acidified with 1 or 2 drops of 1 N hydrochloric or sulfuric acid per 100 cc.

0.5 M CO_2-Free Na_2HPO_4.—17.9 gm. of $Na_2HPO_4 \cdot 12\ H_2O$, or 7.1 gm. of anhydrous Na_2HPO_4, made up to 100 cc. The solution should

be made with minimum exposure to air, and at once drawn up into a soda-lime tube (Fig. 1).

Another way of preparation, from the H_3PO_4 and concentrated NaOH described for urine analysis, is also convenient. In a 100 cc. measuring flask place about 50 cc. of water and 3.33 cc. of syrupy H_3PO_4 of specific gravity 1.72 (this is approximately 15 M H_3PO_4). Add 1 drop of phenolphthalein and run in 18 N NaOH till the solution turns pink. At once dilute to 100 cc., mix, and draw up into a soda-lime tube.

10 per cent Urease in Glycerol-Phosphate Solution.—Jack bean urease dissolved in water slowly produced carbon dioxide, sufficient in the course of an afternoon to increase by several millimeters the pressure reading in a blank analysis. When the enzyme is dissolved in 50 per cent glycerol CO_2 formation does not occur. Phosphate also is added to the solution, to neutralize the acidity of the Folin-Wu filtrate and provide a proper pH for the action of the urease.

Place 1 gm. of Squibb's urease in a 10 cc. measuring cylinder, and stir into it 2 cc. of water. When the mixture has become homogeneous add 3 cc. of the 0.5 M Na_2HPO_4 solution, followed by 5 cc. of glycerol. Mix, and at once draw up into a 10 cc. burette protected at the top by a soda-lime tube. The delivery tube of the burette below the cock should be 7 or 8 cm. long, and should be provided with a rubber tip to permit delivery through a mercury seal directly into the chamber of the gas apparatus, in the manner recently described (4).

5 N NaOH Solution.—27 cc. of the saturated NaOH solution, described for urine analysis, are diluted to 100 cc. and drawn into a soda-lime tube (Fig. 1).

1 N Lactic Acid (Approximate).—1 volume of commercial lactic acid of 1.20 specific gravity is diluted to 10 volumes with water.

0.4 per cent brom-thymol blue, described above for urine analyses.

Caprylic-ethyl alcohol mixture, 1 to 4, described for urine analyses.

Procedure A, for Determinations in a Series of Blood Filtrates.

Preliminary Aeration of Filtrate and Control Solution.—Prepare the filtrates as described by Folin and Wu (1).[2] Collect each filtrate in a

[2] Instead of diluting the blood with water and then adding 1 cc. each of $\frac{2}{3}$ N sulfuric acid and 10 per cent sodium tungstate, a convenient simplification for

flask of volume equal to at least 10-fold that of the filtrate, and add a drop of 0.4 per cent solution of brom-thymol blue for each 10 cc. The filtrate must show a clear yellow acid color. To remove preformed CO_2 derived from the blood bicarbonate, shake each flask, *unstoppered*, vigorously with a horizontal, whirling motion for 15 seconds. Insert a tube into the flask, and draw fresh air through it for a few seconds. Repeat this process twice more. Each extraction removes about 90 per cent of the CO_2 present, so that the last leaves only a negligible thousandth of that originally present. Several flasks may be held in the hand and shaken together, when a series of analyses is being performed.

Treat a similar volume of acidified 0.9 per cent NaCl solution in the same manner, with the precaution to ascertain that the solution is *within 0.2°C. of the temperature of the filtrates.* A variation of 1° would change the dissolved air content by 0.003 volume per cent, and affect the obtained blood urea nitrogen content by 0.5 mg. per 100 cc.

Transfer of Sample to Gas Apparatus and Digestion with Urease.— With a 5 cc. pipette, provided with a rubber tip as described by Van Slyke and Neill, and calibrated to deliver between two marks, transfer 5 cc. of filtrate to the chamber of the blood gas apparatus. Before the transfer a small drop of caprylic-ethyl alcohol is drawn into the capillary below the cup, into which are then poured 3 or 4 cc. of water and 0.5 to 1 cc. of mercury. The tip of the delivering pipette is sealed by immersion in the mercury, so that the solution is delivered from the pipette into the chamber of the gas apparatus without danger of admixture with either air bubbles or water (4). It is convenient, though not obligatory, to use a pipette provided with a stop-cock.

0.5 cc. of the urease solution, measured from the burette, is then run into the chamber under the mercury seal in the same manner. Before the tip of the burette is lowered into the mercury it is washed in the water in the cup to remove the drop of urease-phosphate at the tip, which may have absorbed some CO_2 from the air. The urease and

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routine analyses is to use a single solution containing the water, tungstate, and acid. It is prepared by adding 1 volume of the 10 per cent tungstate to 8 volumes of N/12 sulfuric acid. Enough of this is prepared to last about a fortnight. If it stands longer a precipitate is likely to form in it. In that case it is discarded. 1 volume of blood is mixed with 9 volumes of this solution.

filtrate are mixed by lowering and raising the mercury in the chamber a few centimeters, and are permitted to react for 1 or more minutes. 1 minute is sufficient with the Squibb's urease we have used, of which 100 mg. at pH 6.8 to 7.2 and a temperature of 20° will decompose 9.5 mg. of urea per minute. The mixture should turn to a greenish color. If it remains yellow, because of unusual acidity of the filtrate, another 0.5 cc. of urease-phosphate solution may be added.

Determination of CO_2 Formed from Urea.—After the urease has acted 1 minute 0.20 cc. of 1 N lactic acid is run under the mercury seal into the chamber from a rubber-tipped burette in the same manner as the sample and urease. The chamber is evacuated and shaken 1½ minutes. The gas volume is reduced to 0.5 cc. for ordinary blood, but only to 2.0 cc. for blood with high urea content. The technique described on p. 533 of Van Slyke and Neill's paper for adjusting the gas volume in CO_2 determinations is to be followed. In case one attempts first to reduce the volume to 0.5 cc., but finds the gas pressure too high to read in the manometer, the mercury in the chamber must be lowered again to the 50 cc. mark, the solution shaken for another half minute, and the gas volume brought to 2.0 cc. With the gas volume at either 0.5 or 2.0 cc., the pressure p_1 is read on the manometer.

5 cc. of 0.9 per cent NaCl solution are analyzed in the same manner. The reading is p_0.

The pressure of CO_2 from urea is $P_{urea} = p_1 - p_0$ as in the urine analysis described above.

In case the temperature recorded by the thermometer in the gas apparatus changes during the interval between the analysis of the blank and that of the filtrate, 1.3 mm. is added to p_0 as in the urine analysis, for each 1° rise when the pressure is measured with the gas at 2 cc. volume. When the gas pressure is measured with the gas at 0.5 cc. volume the correction is 1.7 mm. per 1° temperature change. This correction is added to p_0 if the filtrate is analysed with the gas apparatus at a higher temperature than the blank, and subtracted if the reverse occurs.

Procedure B, for Determinations in Single Samples of Blood Filtrate.

Removal of Preformed CO_2.—5 cc. of filtrate proceded by a drop of caprylic-ethyl alcohol are delivered into the gas apparatus under a

mercury seal as described above. To remove CO_2 the chamber is evacuated and shaken 30 seconds. Mercury is admitted from below until the chamber is about one-quarter full. Then *without stopping the inflow of mercury, the upper cock is opened, admitting air into the chamber.* The admission of mercury into the chamber is continued until all the gases have been completely driven out through the cock above. The air is admitted in order to dilute the CO_2 in the chamber and prevent its reabsorption by the blood solution. If the upper cock were kept closed until the extracted CO_2 had been compressed at the top of the chamber, some of the extracted CO_2 gas would go back into solution. The above procedure removes 90 per cent of the preformed CO_2 from the blood. It is repeated twice, making three extractions in all, which leave only a negligible 0.001 of the preformed CO_2.

Digestion with Urease.—0.5 cc. of the urease-phosphate solution is measured into the cup of the apparatus, through a mercury seal, as in Procedure A. The urease is mixed with the filtrate in the chamber by once lowering the mercury to the bottom of the chamber. The mixture is allowed to stand 1 minute for completion of the action of the urease. It should turn from clear acid yellow to a green or blue color, indicating that the phosphate has neutralized the acidity of the filtrate. If the solution does not turn green, add a larger amount of urease-phosphate.

Determination of CO_2 Formed from Urea.—0.20 cc. of 1 N lactic acid is added through the mercury seal; the gases are extracted and their pressure, p_1, read at either 0.5 or 2.0 cc. volume, as described above for Procedure A.

To absorb the CO_2 5 or 6 drops of 5 N NaOH are admitted into the chamber. The cock leading to the leveling bulb is then opened to permit the solution to rise for a moment into the upper stem of the chamber. The gas volume is brought again to the original 0.5 or 2.0 cc., and the manometer reading p_2 is taken. The pressure P_{urea} due to CO_2 formed from urea is

$$P_{urea} = p_1 - p_2 - c$$

where c is the value of $p_1 - p_2$ obtained in a blank analysis performed on 5 cc. of acidified water in the place of blood filtrate. The c value,

due to traces of CO_2 in the enzyme and phosphate solution, should be only a few millimeters with the gas volume at 0.5 cc.

Washing the Apparatus between Analyses.—Before each analysis the gas apparatus is washed twice with 5 to 10 cc. of distilled water, as described on p. 534 of Van Slyke and Neill's paper.

Direct Determination in Whole Blood or Plasma.

As in Procedure B described above for blood filtrate, the entire operation, including removal of preformed CO_2 and digestion with urease, is carried out in the gas apparatus. Two procedures will be described, for the analyses of 1.0 and 0.2 cc. samples respectively.

Reagents for Direct Analysis of Whole Blood or Plasma.

The reagents required are the *5 N NaOH* and *1 N lactic acid,* described above for analysis of the Folin-Wu filtrate, plus the following.

0.1 N Lactic Acid.—The approximately 1 N lactic acid is diluted 10-fold, and is standardized by titration against standard alkali. Water or 1 N lactic acid is then added in amount necessary to bring the concentration to 0.1 N. A margin of error of 3 per cent may be allowed.

0.02 N lactic acid (for micro blood analysis). The 0.1 N acid is diluted 5-fold.

0.26 M Na_3PO_4 Solution.—In a 100 cc. flask place 175 cc. of syrupy phosphoric acid of specific gravity $D\frac{20°}{20°} = 1.72$ to 1.73. This phosphoric acid is of approximately 15 M H_3PO_4 concentration. Fill the flask half full with water, add 2 drops of 1 per cent phenolphthalein solution, and titrate with the carbonate-free, saturated (approximately 18 N) NaOH solution from a 10 cc. burette until the solution in the flask turns red. At this point 2 molecules of NaOH have been added per molecule of H_3PO_4, and Na_2HPO_4 has been formed. Note the volume of NaOH solution used and add exactly half as much more, to change the phosphate to Na_3PO_4. The total volume of 18 N NaOH required should be about 4.3 cc. Immediately after the addition of the alkali has been completed, and before the alkaline phosphate solution has had opportunity to absorb CO_2 from the air, dilute the solution to the 100 cc. mark, stopper the flask, mix the solution, and

draw it up into a soda-lime tube (Fig. 1), from which it may be measured by drops for analyses.

10 Per Cent Urease Solution in 50 Per Cent Glycerol.—1 gm. of Squibb's preparation of jack bean urease prepared by the acetone precipitation method of Van Slyke and Cullen (6) is dissolved in 5 cc. of water, and 5 cc. of glycerol are mixed with the solution. The enzyme solution should be prepared the same day it is used. It need not be protected from air, as it is too acid to absorb CO_2. It is not expedient to mix it with the Na_3PO_4 prior to use, because the alkalinity of the phosphate destroys the enzyme rather rapidly.

Determination in 1 Cc. of Whole Blood or Plasma.

Removal of Preformed CO_2 from Blood.—Place a drop of caprylic-ethyl alcohol in the cup of the blood gas apparatus, and draw it down into the capillary beneath the cup. Then place 1 cc. of 0.1 N lactic acid in the cup, and add water up to the 4.5 cc. mark. From a stop-cock pipette run 1 cc. of blood under the water solution into the chamber, and then draw the acidified water also into the chamber. If a bubble of air also enters the chamber no harm is done; it will be ejected later with the preformed CO_2. The preformed CO_2 is now removed by three successive extractions of 30 seconds each, in the manner described above for Procedure B with the Folin-Wu filtrate.

Digestion of Blood with Urease.—After removal of preformed CO_2, place in the cup of the apparatus 1 cc. of 10 per cent urease solution, and run into the urease solution 0.25 cc of the 0.26 M Na_3PO_4 solution, measured by drops from the soda-lime tube (the first 2 or 3 drops are to be discarded as they have absorbed atmospheric CO_2). The urease-phosphate mixture is at once drawn down into the chamber of the apparatus, and the cock is sealed with a drop of mercury. In order to mix urease and blood, and to bring the enzyme into contact with the portions of blood solution wetting the walls of the chamber, the mercury in the latter is lowered to the bottom and then permitted to rise again. The mixture is now permitted to stand a sufficient length of time (1 minute with Squibb's urease) for the enzyme to complete its action.

Extraction and Measurement of CO_2 Formed from Blood Urea.—After the enzyme has finished its action 0.5 cc. of 1 N lactic acid is

placed in the cup, and 0.25 cc. is drawn down into the apparatus, making the total volume of solution up to 7 cc. The cock is sealed with mercury, the chamber is evacuated, and is shaken 2 minutes, as in determinations of blood CO_2. The volume of the extracted gas is reduced, ordinarily to 0.5 cc. If the blood urea content is over 75 mg. per 100 cc. (urea nitrogen over 35 mg.), however, the CO_2 pressure at 0.5 cc. volume will exceed 400 mm. With such bloods the volume of the extracted gas is reduced only to 2 cc. The technique described by Van Slyke and Neill, on p. 533 of their paper, for reducing the volume of extracted CO_2 for pressure measurement is to be followed.

With the extracted gases at 0.5 or 2 cc. volume, the manometer reading p_1 is noted. Without releasing the vacuum, 3 or 4 drops of 5 N NaOH solution are admitted, a drop at a time, from the cup to absorb the CO_2. The vacuum is then released and the solution is permitted to rise for a moment into the upper stem of the chamber, to wash out the alkali. The mercury in the chamber is finally lowered again until the surface of the blood solution has fallen to the 0.5 or 2 cc. mark used for the first reading, and the second manometer reading, p_2, is taken.

A control analysis is carried through, in which the blood is replaced by water solution. The difference between p_1 and p_2 in the control analysis is designated as c, for the calculation. The value of c is usually 5 or 6 mm. with a gas volume of 0.5 cc., and 1 or 2 mm., with a gas volume of 2 cc. The pressure due to CO_2 from urea in the analysis is

$$P_{\text{urea}} = p_1 - p_2 - c$$

The CO_2 pressure c observed in the control analysis is due in part to a trace of CO_2 in the alkaline phosphate, in part to the trace of CO_2 present in the water used to dissolve the urease.

A total solution volume as great as 7 cc. for 1 cc. of blood is used because the 5 N sodium hydroxide can be run into it for absorption of CO_2 at the end of the analysis. If only a 3-fold dilution of whole blood were used, the 5 N alkali would cause a gummy precipitate of hemoglobin on the walls of the chamber. One would have to use 1 N alkali, which would have to be freed of air before it could be used.

Before each analysis the chamber is washed once with dilute lactic acid, as described on p. 534 of Van Slyke and Neill's paper.

Determination in 0.2 Cc. of Whole Blood or Plasma.

If the blood is drawn by skin puncture outside the laboratory a convenient procedure is to place 1 cc. of 0.02 N lactic acid in a small test-tube (6 or 7 mm. inner diameter) and draw 0.2 of blood directly into capillary pipette. The pipette is emptied into the lactic acid, and is rinsed twice by drawing the acid up into it. The blood solution, together with a drop of caprylic-ethyl alcohol, is transferred to the chamber of the blood gas apparatus, 0.6 cc. of water, in portions of 3 or 4 drops each, being used to wash adherent drops of blood solution from the test-tube into the chamber of the blood gas apparatus.

If the blood is drawn in the laboratory, the procedure may be simplified by emptying the pipette directly into the blood gas apparatus. The 1 cc. of 0.02 N lactic acid, plus 0.6 cc. of water, is then used to rinse the blood adherent to the pipette and the cup of the apparatus into the chamber.

After the 0.2 cc. of blood and the acid, by either of the above procedures, have been brought into the chamber, the CO_2 is removed by extracting three times, as described above. Then 0.2 cc. of the 10 per cent urease is placed in the cup of the apparatus, together with 0.05 to 0.07 cc. of the 0.26 M Na_3PO_4 (2 drops), and the mixture is drawn down into the chamber, making the total volume of solution approximately 2 cc. The enzyme-phosphate solution is mixed with the blood by lowering the mercury to the bottom of the chamber and permitting it to rise again. The urease is allowed to act on the urea for 1 minute (or longer if a weaker enzyme necessitates it). Finally the mixture is acidified by admission of 2 or 3 drops of 1 N lactic acid, the CO_2 is extracted by 2 minutes shaking of the evacuated chamber, and the pressure is measured, p_1, with the gas volume at 0.5 cc. The vacuum is released; 1 or 2 drops of 5 N sodium hydroxide are admitted to absorb the CO_2, the meniscus of the solution is lowered again to the 0.5 cc. mark, and pressure p_2 is read on the manometer.

A control determination is performed in which the blood is replaced by water. The difference between p_1 and p_2 in the control analysis is designated as c. The pressure of CO_2 from urea is

$$P_{urea} = p_1 - p_2 - c$$

Before each determination the apparatus is washed twice with distilled water.

Calculation of Results of Analyses.

The factors by which the P_{urea} values obtained in the above analyses are multiplied in order to give the urea contents of urine or blood are given in Tables I, II, and III.

TABLE I.

For Analysis of Urine.

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied.

Volume of solution extracted = 3.5 cc. Gas volume at which pressure is measured = 2.000 cc.

Temperature.	Factors giving gm urea N per 100 cc. urine		Factors giving gm urea per 100 cc urine.	
	Sample = 0 1 cc. urine	Sample = 0 2 cc urine	Sample = 0 1 cc. urine	Sample = 0 2 cc urine.
°C.				
15	0 003443	0 001723	0 00738	0 003689
16	24	13	34	68
17	04	08	30	47
18	0 003386	0 001693	26	27
19	68	84	22	08
20	51	76	18	0 003589
21	33	68	14	70
22	14	59	10	51
23	0 003297	50	07	33
24	81	41	03	15
25	65	38	00	0 003497
26	50	26	0 00697	80
27	34	18	93	64
28	19	11	90	49
29	03	03	86	32
30	0 003188	0 001595	83	16
31	74	88	80	01
32	60	81	77	0 003386
33	46	74	74	71
34	32	67	71	56

The factors in the tables were calculated as follows: Since 1 mol of urea yields 1 mol of CO_2 the molecular concentrations of $CO(NH_2)_2$ or urea N_2 are calculated, from the CO_2 pressures observed in the gas

TABLE II.

For Analysis of Folin-Wu Blood Filtrate.

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied.

Volume of solution extracted (S) = 5.7 cc. Sample = 0.5 cc. blood. α' = 0.945 α' for water.

Temperature	Factors giving mg. urea N per 100 cc. blood		Factors giving mg. urea per 100 cc. blood.	
	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.
°C				
15	0.720	0.1834	1.540	0.393
16	15	21	30	90
17	10	09	20	88
18	06	0.1798	10	85
19	01	87	00	83
20	0.697	76	1.491	80
21	93	65	82	78
22	88	54	73	76
23	84	43	64	73
24	80	33	55	71
25	76	23	47	69
26	72	13	39	67
27	68	03	31	65
28	65	0.1694	23	63
29	61	85	15	61
30	58	76	07	58
31	54	68	00	57
32	51	59	0.1393	56
33	48	51	87	54
34	45	43	80	52

apparatus, by the same equation used for CO_2 (see Van Slyke and Sendroy (8)); *viz.*,

$$\text{mM } CO_2 \text{ or urea per liter} = P_{\text{urea}} \times \text{factor}$$

$$\text{Factor} = \frac{1000}{\text{cc. sample}} \times \frac{i\,a}{760 \times 22.26\,(1 + 0.00384\,t)} \times \left(1 + \frac{S}{A - S}\,\alpha'\right)$$

P is the pressure of CO_2 (from urea in the present case). a is the volume of the gas, 2.0 or 0.5 cc., at which the pressure is read. i is

the empirical factor correcting for reabsorption of CO_2, found to be 1.017 when a = 2.0 cc., 1.037 when a = 0.5 cc. t is the temperature centigrade. A is the volume of the gas apparatus chamber, usually

TABLE III.

For Direct Analysis of Whole Blood or Plasma.

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied.

Temperature	Factors to give mg urea N per 100 cc blood.			Factors to give mg urea per 100 cc blood		
	Sample = 1 cc. blood		Sample = 0 2 cc blood	Sample = 1 cc blood		Sample = 0.2 cc blood
	a = 0 5 cc S = 7 0 "	a = 2 0 cc. S = 7 0 "	a = 0 5 cc S = 2 0 "	a = 0 5 cc S = 7 0 "	a = 2 0 cc S = 7 0 "	a = 0.5 cc. S = 2 0 "
°C						
15	0 0955	0 3740	0 424	0 2048	0 0802	0 909
16	47	12	22	30	0 0796	05
17	40	0 3684	20	12	90	00
18	33	59	18	0 1998	84	0 896
19	26	34	16	84	79	92
20	19	08	14	70	73	88
21	13	0 3583	12	57	68	84
22	08	58	11	44	63	80
23	02	34	09	31	58	76
24	0 0895	10	07	17	52	73
25	89	0 3488	05	04	48	68
26	83	66	03	0.1891	43	65
27	77	45	02	78	38	61
28	72	24	00	65	34	58
29	68	04	0 398	62	30	54
30	63	0 3384	97	49	25	50
31	58	66	95	36	21	47
32	54	48	93	33	17	44
33	49	29	92	20	13	41
34	44	11	91	07	10	37

a indicates gas volume at which pressure is measured.
S indicates volume of solution from which the CO_2 is extracted in the apparatus.

50 cc.; S is the volume of solution extracted in the apparatus; α' is the Henry distribution coefficient, between gas phase and solution, of the gas determined.

From the millimolar factors obtained as outlined above, the factors for calculating grams of urea and urea nitrogen were obtained by use of the molecular weights, 60.05 for $CO(NH_2)_2$ and 28.016 for N_2.

For the Folin-Wu filtrate analyses, it was found that in the mixture extracted, *viz.* 1 volume of filtrate plus 0.1 volume of urease solution in 50 per cent glycerol plus 0.04 volume of 1 N lactic acid, CO_2 had a solubility only 0.945 times the solubility of CO_2 in water. Hence in calculating the factors for the Folin-Wu filtrate analyses the α' values of Table I of Van Slyke and Neill have been multiplied by 0.945. For the other analyses, in which blood or urine was diluted with water containing relatively little salt, the α' values of water were used.

Gasometric Standardization of Urease Activity.

In previous papers (5, 9) on the mode of action of urease it has been shown that the maximum activity of the enzyme is exerted at a pH of approximately 7, maintained by phosphate buffers, and a high concentration of urea. To determine the activity of urease Van Slyke and Cullen (6) caused the enzyme to act on a solution containing 0.25 M KH_2PO_4, 0.25 M K_2HPO_4 or Na_2HPO_4, and 1 M urea concentrations. They permitted 100 mg. of urease (1 cc. of 10 per cent solution) to act on 5 cc. of this solution at 20° for 15 minutes, then stopped the enzyme action by adding excess K_2CO_3, and determined by aeration the amount of ammonia formed. They specified that for use in their method the ammonia should neutralize at least 8 cc. of 0.1 N acid, indicating 24 mg. of urea decomposed in 15 minutes, or 0.016 mg. of urea decomposed per minute by 1 mg. of urease.

The present Squibb's urease, made according to Cullen and Van Slyke's method, but from jack beans instead of soy beans, has from 3 to 6 times this activity: it can decompose at 20° from 0.05 to 0.10 its weight of urea per minute.

The activity is determined in the gas apparatus as follows: 2 cc. of the above phosphate-urea solution of Van Slyke and Cullen (6) at room temperature are run into the chamber of the apparatus. 1 cc. of water is placed in the cup, and 0.1 cc. of 5 per cent urease, containing 5 mg. of enzyme, is run underneath the water from a

capillary pipette. The enzyme solution, followed by 0.9 cc. of water, is run into the chamber. The time is noted. The intermixture of enzyme and urea solution is quickly completed by lowering the mercury in the chamber a few centimeters and letting it rise again. After an interval, usually 5 minutes, sufficient to yield 200 to 400 mm. of CO_2 pressure, 0.5 cc. of 1 N lactic acid is run into the chamber, the CO_2 is extracted, and the manometer reading, p_1, is taken with the gas volume at 2.0 cc. The apparatus is washed out twice with water, and a control analysis is run without enzyme, the reading obtained being p_0.

The number of milligrams of urea that 1 mg. of enzyme preparation can decompose in 1 minute is found by multiplying $(p_1 - p_0)$ by the factor in Table IV corresponding to the temperature, and dividing by the number of minutes the enzyme acted and the number of milligrams of enzyme present.

$$\text{Mg. urea split per minute at } 20^\circ \text{ by 1 mg. urease} = \frac{(p_1 - p_0) \times \text{factor}}{(\text{minutes action}) \times (\text{mg. urease})}$$

Example.—In standardizing the enzyme used in the present work the $p_1 - p_0$ value was 396 mm. at 22.5°, 5 mg. of the enzyme having acted 5 minutes. Inserting these values into the above formula we obtain:

$$\text{Mg. urea split per minute at } 20^\circ \text{ by 1 mg. urease} = \frac{396 \times 0.0060}{5 \times 5} = 0.095$$

Calculation of Factors in Table IV.—The $(p_1 - p_0)$ value obtained in the above standardization test, multiplied by a factor from the fourth column of Table I indicates the number of milligrams of urea decomposed. (This factor gives gm. of urea per 100 cc. of urine when 0.1 cc. is analyzed. Gm. per 100 cc., however, is the same as mg. in the 0.1 cc. sample analyzed.) However, if the temperature is above 20°, the activity of the enzyme is increased; *e.g.*, at 30° it decomposes twice as much urea per minute as at 20°. Hence a correction factor must be introduced to bring the figure to the value it would have at 20°. Van Slyke and Cullen (5) found that between 10° and 50° the effect of temperature on urease activity is indicated by the equation:

$$\text{Log} \frac{\text{activity at } t_1^\circ}{\text{activity at } t_2^\circ} = 0.029\ (t_1 - t_2)$$

where t_1 and t_2 are any two temperatures within the above range. If t_1 is 20° and t_2 is t°, the temperature of the analysis, this equation becomes

$$\text{Log } \frac{\text{activity at } 20^\circ}{\text{activity at } t} = 0\ 029\ (20 - t)$$

TABLE IV

For Calculating Activity of Urease

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied to Give Milligrams of Urea That Would be Decomposed at 20°

Temperature	Factor
°C	
15	0 0103
16	0 0096
17	89
18	85
19	77
20	72
21	68
22	62
23	58
24	54
25	50
26	47
27	43
28	40
29	37
30	35
31	33
32	30
33	28
34	26

which may also be expressed as

$$\frac{\text{Activity at } 20^\circ}{\text{Activity at } t^\circ} = 10^{0\ 029\ (20 - t)}$$

Hence each factor in the fourth column of Table I is multiplied by $10^{0\ 029(20-t)}$ in order to correct for the effect of temperature on

the activity of the enzyme. The resulting combined factors are those of Table IV. They are carried out only to two places, because the velocity determinations performed as described are not sufficiently accurate to justify a third figure.

EXPERIMENTAL.

Urine Analyses.

In order to test the urine method on known solutions, Merck's urea was made up in solutions of 1, 2, and 3 per cent concentration. These were analyzed by macro-Kjeldahl determinations performed on 3 cc. samples, also by the Van Slyke-Cullen method with 0.5 cc. samples, and by the present gasometric method, with samples equivalent to 0.1 cc. of urine. The results, in Table V, represent the averages of closely agreeing triplicate estimations.

TABLE V.

Analyses of Pure Urea Solutions by the Urine Urea Methods.

Urea added to solution		Urea N determined		
Urea	Urea N	Kjeldahl	Van Slyke-Cullen urease method	Present gasometric urease method
gm per 100 cc	*gm per 100 cc*	*gm per 100 cc*	*gm. per 100 cc*	*gm per 100 cc*
1 00	0 467	0 465	0 463	0 465
2 00	0 934	0 931	0 918	0 930
3 00	1 867	1 860	1 834	1 868

A number of urines were analyzed both by gasometric determination of the CO_2 from the urea, and by the Van Slyke-Cullen procedure for aerating and titrating the ammonia. The results are given in Table VI.

Blood Analyses.

Time Required for Urease Action.—To 30 cc. of horse blood, containing 11.4 mg. of urea nitrogen per 100 cc., 1 cc. of 6 per cent urea solution was added, in order to raise the urea content to that encountered in uremic blood. The calculated urea nitrogen content of the mixture was 101.4 mg. per 100 cc. Analyses were performed on 1

TABLE VI.

Comparison of Urea Determinations in Urine by the Van Slyke-Cullen Ammonia Titration Method and the Present Gasometric CO_2 Method.

Urine No.	Gasometric measurement of CO_2.				Van Slyke-Cullen titration of NH_3.		
	Urine volume in sample used for determination.	Observed pressure at 2 cc volume of CO_2 from urea.	Temperature.	Urea N per 100 cc. urine.	0 02 N HCl required in titration.	Urine volume used for analysis.	Urea N per 100 cc. urine.
	cc.	*mm.*	°*C.*	*gm*	*cc.*	*cc.*	*gm.*
1	0.1	276 4	19 0	0 931	16 52	0.5	0.925
	0 1	275 6	19.0	0 928	16 56	0.5	0.927
2	0.1	283.4	19 0	0.955	16 82	0.5	0.942
	0 1	283.8	19 0	0.956	16.80	0.5	0.941
3	0 2	274 2	19 7	0.460	8.11	0 5	0 459
	0 2	271 9	19 7	0 456	8 16	0.5	0 464
4	0 2	255 4	19 9	0 428	7 49	0.5	0 419
	0 2	256 6	19 9	0 428	7 58	0.5	0 429
5	0 2	88.8	19.4	0 149	2 58	0 5	0.145
	0 2	90 5	19 5	0.152	2 53	0 5	0.142
6	0 2	100 1	19.9	0 168	2 94	0 5	0.165
	0 2	101 2	20 4	0 169	2 91	0.5	0 163
7	0 1	104 8	22 0	0 347			
	0 1	104.8	22 0	0 347			
	0 2	207 7	20.5	0 347			
	0 2	207.0	20 5	0 346	6.16	0 5	3 45
	0 2	207.7	21.5	0 346	6.20	0 5	3.47
8	0 1	117.8	21.8	0 391			
	0 2	235 6	20 8	0 393			
	0 2	235.2	20 5	0 393	6.96	0.5	3.90
	0 2	236.7	21 5	0.394	6 96	0.5	3.90
9	0 1	64 8	22 5	0 214			
	0.2	126.6	21 0	0 211			
	0 2	126 2	20.5	0 211	3.77	0 5	2.11
	0 2	126 4	21 5	0.210	3.83	0.5	2.15

cc. samples by the procedure described above for whole blood. The period permitted for action of the urease, however, was varied by making the final addition of lactic acid at varying intervals after the enzyme and blood had been mixed in the gas apparatus. The urease was that described above in connection with the standardization: 100 mg. (the amount employed) could decompose a maximum of 9.5 mg. of urea in 1 minute at 20°. The action on the blood was so rapid that it was complete within a half minute, which was about the time required, after mixing the blood and enzyme, to measure out and add the acid. The results are given in Table VII, together with those by the Van Slyke-Cullen aeration-titration procedure.

TABLE VII.

Time Required for Action of 1 Cc. of 10 Per Cent Urease on 1 Cc. of Uremic Blood.

Gasometric determination on 1 cc blood					Van Slyke-Cullen titration of NH_3 from 3 cc blood	
Period of digestion with urease.	Pressure of CO_2 from urea, measured at 2 cc volume	Temperature	Factor	Urea N per 100 cc blood	0 01 N acid neutralized	Urea N per 100 cc blood
min	*mm*	°C.		*mg*	*cc.*	*mg*
0 5	278 8	19 0	0 3634	101 4	21 89	102 1
					21 92	102 3
1.0	275 8	18.5	0 3646	100 6		
2 0	276 9	18 6	0 3644	100 9		
4 0	277 5	19 0	0 3634	100 9		

Analyses of Standard Urea Solutions.—By dilution of a stock 1 per cent urea solution, 0.200, 0.100, and 0.050 per cent urea solutions were prepared. They were analyzed by the procedures described above for blood urea, and also by the Van Slyke-Cullen procedure. For greater accuracy, the latter was performed on 5 cc. samples instead of the 2 or 3 cc. portions usually employed in routine blood analyses. The results are given in Table VIII.

Analyses of Nephritic Bloods.—In Tables IX and X are given data from a number of nephritic bloods, showing varying degrees of urea retention, with the results of urea determinations by the gasometric methods described above. Parallel results by the Van Slyke-Cullen method are also given. The agreement between the results obtained

TABLE VIII.

Comparison of Results of Analyses of Standard Urea Solutions by Gasometric Blood Urea Methods and by Van Slyke-Cullen Method.

Solution.	Method	Gasometric measurement of CO_2.						Titration of NH_3.	
		Volume of solution equivalent to sample.	Gas volume at which CO_2 pressure was measured.	Pressure of CO_2 from urea.	Temperature.	Factor.	Urea N per 100 cc.	0.01 N HCl neutralized by NH_3 from 5 cc. solution.	Urea N per 100 cc.
		cc.	*cc*	*mm.*	*°C.*		*mg*	*cc*	*mg*
Urea solution.	Whole	1 000	2 000	257 0	19.0	0 3634	93.5	32.78	91.8
200 mg. urea,	blood.	1 000	2 000	257.1	19 7	0.3616	93 0	32 55	91.2
93 3 mg. N,									
per 100 cc.	Filtrate	0 500	2.000	135 1	21.1	0 693	93 6		
	A.	0.500	2 000	134.1	21.1	0 693	92 9		
		0.500	2 000	134.0	21 1	0 693	92.9		
	Filtrate	0.500	2 000	134 2	21.0	0.693	93 0		
	B.	0 500	2.000	133.8	21 0	0 693	92.8		
Urea solution.	Whole	1 000	2 000	127 6	20 0	0 3608	46 1	16 34	45.8
100 mg. urea,	blood.	1 000	2.000	128 0	20.0	0 3608	46 2	16.42	46 0
46 7 mg. N,		1.000	2.000	127 5	20 0	0 3608	46 0		
per 100 cc.									
	Filtrate	0.500	2 000	69.0	21 2	0.692	47.1		
	A.	0.500	2 000	68 3	21.2	0.692	46 5		
		0.500	2 000	68 5	21.2	0.692	46.7		
		0.500	0.500	266 7	21 2	0 1763	47.0		
		0 500	0.500	261.5	21.2	0.1763	46 1		
		0.500	0.500	265 0	21.2	0 1763	46 7		
	Filtrate	0.500	0.500	262.4	21.0	0.1765	46.3		
	B.	0 500	0.500	263.8	21 0	0 1765	46 6		
Urea solution.	Whole	1.000	2.000	64.7	20.0	0.3608	23.3	8.09	22.7
50 mg. urea,	blood.	1.000	2.000	64.4	20 0	0.3608	23 2	8.09	22.7
23 3 mg. N,		1.000	0.500	249.7	20 0	0.0919	22.9		
per 100 cc.		1.000	0.500	249.6	20.0	0.0919	22.9		
	Filtrate	0.500	0.500	133.3	21.2	0.1763	23.5		
	A.	0 500	0.500	131 5	21.2	0.1763	23.2		
		0.500	0.500	131.7	21.2	0.1763	23.2		
	Filtrate	0.500	0.500	131.8	21.0	0.1765	23.3		
	B.	0 500	0 500	133 0	21.0	0.1765	23.5		

by NH_3 and CO_2 determination respectively affords evidence of the specificity of the enzyme for urea, among the blood constituents.

TABLE IX.

Comparison of Blood Urea Determinations by Gasometric Methods and by Van Slyke-Cullen Titration Method.

Blood No	Gasometric measurement of CO_2.							Titration of NH_3.	
	Method	Volume of blood equivalent to sample.	Gas volume at which CO_2 pressure was measured	Pressure of CO_2 from urea.	Temperature	Factor.	Urea N per 100 cc blood.	1 025 N HCl neutralized by NH_3 from 2 cc blood	Urea N per 100 cc. blood.
		cc	*cc.*	*mm.*	*°C.*		*mg*	*cc*	*mg.*
1	Whole blood.	1 000	0 500	283.9	24 5	0 0892	25 3	3.49	24.4
	" "	1 000	0 500	280 3	23 1	0 0902	25.3	3 60	25 2
2	Whole blood.	1 000	0 500	69 7	23 5	0 0898	6 26	0 88	6.2
	" "	1 000	0 500	70 8	23 7	0 0897	6 35	0 98	6 9
3	Whole blood.	1 000	0 500	379 7	24 0	0 0895	34 0	4 85	34.0
	" "	1 000	0 500	376 7	24 0	0 0895	33 7	4 74	33.2
4	Whole blood.	1 000	2 000	147.4	24 5	0.3499	51 6	7 36	51.6
	" "	1.000	2 000	147 4	24.5	0 3499	51 6	7 42	51.9
5	Filtrate A.	0 500	0.500	150 8	23 5	0.1738	26.2		
	" "	0 500	0 500	151 8	23 5	0 1738	26 3		
	Whole blood.	1.000	0 500	301.4	23 0	0 0902	27 2	3 80	26 6
	" "	1 000	0 500	301 2	23 0	0 0902	27 2	3 81	26.7
6	Filtrate A.	0 500	0 500	146 4	23 5	0 1738	25 4		
	" "	0 500	0 500	146 3	23 5	0.1738	25.4		
	Whole blood.	1 000	0 500	291 0	24 0	0 0895	26 0	3.62	25 3
	" "	1 000	0 500	290 3	24 0	0 0895	26 0	3 64	25.5
7	Filtrate A.	0 500	0 500	75.1	22 5	0.1748	13 1		
	" "	0 500	0 500	74.6	22 5	0 1748	13.0		
	Whole blood.	1.000	0 500	139 2	23.0	0 0902	12 55	1.76	12.3
	" "	1 000	0.500	138 4	23.0	0.0902	12 48	1.76	12 3

TABLE IX—*Concluded.*

Blood No.	Gasometric measurement of CO_2							Titration of NH_3	
	Method	Volume of blood equivalent to sample	Gas volume at which CO_2 pressure was measured	Pressure of CO_2 from urea.	Temperature.	Factor.	Urea N per 100 cc. blood.	1 025 N HCl neutralized by NH_3 from 2 cc. blood.	Urea N per 100 cc. blood
		cc	*cc*	*mm*	°C.		*mg.*	*cc*	*mg*
8	Filtrate A.	0 500	2 000	70 2	22 3	0.687	48 9		
	" "	0 500	2 000	69 7	22 3	0 687	48.6		
	Whole blood.	1 000	2 000	135 8	22 8	0 3530	48 0	6 76	47.4
	" "	1 000	2 000	135 7	22 8	0 3530	48 0	6 76	47.4
9	Filtrate A.	0 500	0 500	127 7	23 1	0 1742	22 2		
	" "	0 500	0 500	127 4	23 1	0.1742	22 2		
	Whole blood.	1 000	0 500	251 0	24 0	0 0895	22 5	3 19	22 3
	" "	1 000	0 500	250 0	24 0	0 0895	22 4	3 26	22 8

TABLE X.

Urea Content of Nephritic Blood by Microgasometric Method.
Blood Sample of 0 2 Cc.

Blood No	Pressure of CO_2 at 0 5 cc volume	Temperature	Factor	Urea N per 100 cc blood.	Urea N per 100 cc blood by Van Slyke-Cullen method.
	mm	°C.		*mg*	*mg*
1	94 9	23 0	0 409	38 8	39 0
2	91 1	23 0	0 409	37 3	36 9
	91.1	23 0	0 409	37 3	
3	99 2	22 3	0 410	40 7	40 7
4	91 6	23 0	0 409	37 5	37.5
5	85.6	22 5	0 410	35 1	35.8
6	104 2	23 0	0.409	42.6	43.4
	104.2	23.0	0.409	42.6	
7	103 9	22 5	0 410	42.6	41 7
	103 0	22 5	0 410	42.2	

SUMMARY.

Methods are described for rapid determination of urea in blood and urine by measuring in the manometric blood gas apparatus the CO_2 of the ammonium carbonate formed by action of urease. The blood urea may be determined in either whole blood, serum, or Folin-Wu filtrate. Micro determinations may be performed with 0.2 cc. of blood.

The methods here presented were developed with the technical assistance of John Plazin.

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[Reprinted from The Journal of Clinical Investigation, June 20, 1927, Vol. iv, No. 2, pp. 235–251.]

THE EXCRETION OF ALBUMIN AND GLOBULIN IN NEPHRITIS.

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(Received for publication, January 14, 1927)

The present work was undertaken in order to ascertain whether the nature of the protein mixture excreted in the different types of nephritis is related to the type and severity of the disease.

I. HISTORICAL

A summary of the early literature on the relation of albumin to globulin excretion in the urine of nephritic patients has been given by Senator (1) and by Cloetta (2). A general survey of the literature has been given given more recently by Geill (3). Hoffman was the first to take up the work in detail. Using the early gravimetric methods in 1882 (4) he reported that any albumin : globulin ratio may occur in any type of nephritis, and the value of the ratio is dependent not upon the type of change in the kidney, but on the intensity of the disease processes. A low ratio signified a severe condition, a high ratio a mild one. The ratio was found to rise with recovery in acute nephritis. Lecorché and Talamon (5) found the ratio to decrease with increased severity of the disease. Csatáry (6) found great fluctuations in the albumin : globulin ratio, but made a general statement that he found ratios below 1 in cases of amyloid kidney and above 10 in cases of contracted kidney. In severe cases the ratio fell Cloetta (2) confirmed the work of Csatáry and reported low ratios in acute nephritis, with rise on recovery, and ratios usually over 10 in chronic nephritis. He found no relation between the albumin : globulin ratios in urine and in serum. Joachim (7) reported a low ratio in amyloid kidney, a high ratio in contracted kidney. A rise in the ratio signified a good prognosis while a fall signified a poor prognosis. Paton (8) reported albumin fractions high in chronic nephritis and low in acute cases. He was unable to find high globulins in amyloid kidney, and was unable to form any conclusions on the relation of the urinary ratio to that of plasma. Dreser (9) reported that the ratio has no diagnostic importance. Gross (10) reported that the ratio varied and had no prognostic or diagnostic value. Strauss (11) reported that the ratio had no diagnostic value, and that it was mostly so different from that of serum that one would be led to believe that the protein excretion is a selective process of the glomerulus. Wallis (12) re-

ported a low ratio in functional albuminuria and "leaky kidney," and a ratio of 6 in chronic and in toxic nephritis. Autenrieth (13) found a distinct prognostic value in the albumin : globulin ratio. In agreement with Hoffman (4) he found ratios under 5 to the accompanied by a poor prognosis.

The *relation of urinary protein excretion to plasma protein concentration* has been studied by a number of authors. Kisch (14) showed that when the total protein excretion was less than 1 gram per liter the total plasma protein was over 7 per cent. When the protein excretion increased, the plasma proteins fell below 7 per cent. Linder, Lundsgaard and Van Slyke (15) found that when the protein excretion exceeded 1 gram per day there was a reduction of the total concentration of protein and of the albumin : globulin ratio in the plasma, the plasma loss affecting chiefly the albumin. Kollert and Starlinger (16) found that when the amount of protein excreted exceeded 1 gram per liter of urine the serum protein fell below 8 per cent, and continued to fall with increased excretion. The serum albumin showed a progressive fall in the same manner, with increased protein excretion In the case of globulin, however, it was found that the higher the globulin fraction rose in the serum the higher the percentage of globulin in the proteins excreted in the urine, so that the albumin . globulin ratio in the urine tended to decrease with the fall in the ratio in the serum

II. METHOD FOR THE DETERMINATION OF PROTEINS IN URINE[1]

Albumin and globulin were separated by precipitating the latter with sodium sulfate, as in Howe's (17) technique for plasma protein separation. The separated proteins were determined by the colorimetric method of Autenrieth (13, 18) which depends on the development of the biuret color by proteins treated with copper sulfate and alkali.

The chief disadvantages of the Autenrieth method have been the lack of satisfactory standards, and the tedious technique of precipitating and washing the proteins. We have found that standards may be prepared from solutions of pure biuret. One milligram of Kahlbaum's biuret was found to give a color equal to that produced by 0.924 mgm of either albumin or total urinary protein. This biuret equivalent of the proteins was obtained by comparison of Kjeldahl and colorimetric determinations on a number of urines. Instead of precipitating with heat and acid we have precipitated the proteins with trichloracetic acid, and have centrifuged instead of washing on a filter.

[1] A preliminary note on the method has been published in the *Proc. Soc. Exp. Biol. Med*, 1927, xxiv, 385.

Magnesium sulfate was also tested for the precipitation of the globulins. Saturation with magnesium sulfate was found to give a greater precipitation of protein than did 22 per cent sodium sulfate. The presence of magnesium sulfate also decreases the depth of the color somewhat in the end reaction: it was necessary to remove the magnesium by precipitating the albumin twice with trichloracetic acid.

Since sodium sulfate gave a filtrate with a biuret equivalent of albumin equal to that of total protein, and since this same precipitant is used for the routine separation of the proteins in the plasma by Howe's method, we adopted sodium sulfate for precipitation of the urine globulins.

Reagents:

10 per cent trichloracetic acid solution.

3 per cent sodium hydroxide solution.

30 per cent sodium hydroxide solution.

20 per cent copper sulfate solution, containing 20 grams $CuSO_4 \cdot 5H_2O$ per 100 cc. solution.

44 per cent sodium sulfate solution containing 44 grams anhydrous Na_2SO_4 per 100 cc. solution. This solution is saturated at 37° and must be kept at that temperature to prevent crystallization. The sodium sulfate solution must be neutral to litmus.

Standard biuret solution: Dissolve 0.4000 gram of biuret in distilled water, and dilute to a volume of 150 cc. This solution will keep in the ice box at least a month.

Preparation of urine samples: Adjust a portion of urine (50 to 200 cc.) to a pH of about 7.4; i.e., slightly alkaline to sensitive litmus paper. The reaction may be adjusted with more certainty by removing drops and testing with phenol red. Filter if not perfectly clear. This same specimen can now be used for the precipitation of globulin and for total protein.

Total protein: Measure 2 cc. of the specimen into a graduated centrifuge tube, add an equal volume of 10 per cent trichloracetic acid, mix with a narrow glass rod, and centrifuge 5 minutes. If the volume of precipitate is between 0.2 and 0.6 cc., the amount of protein in it can be read against the standard described below, and the analysis is continued as described in the next paragraph. If the

volume of precipitate is larger or smaller, a second precipitation is performed, with enough urine to yield a precipitate of between 0.2 and 0.6 cc.

Pour off the supernatant fluid, draining as dry as possible. Dissolve the precipitate in about 3 cc. of 3 per cent sodium hydroxide solution and wash into a 10 cc. graduated cylinder with portions of the 3 per cent sodium hydroxide until the volume has reached about 9 cc. Add 0.25 cc. of 20 per cent copper sulfate solution, dilute to 10 cc. with 3 per cent sodium hydroxide. Mix thoroughly by shaking, let stand 10 minutes, centrifuge, and compare the supernatant fluid in a colorimeter against a standard prepared at the same time.

To prepare the standard color solution measure 5 cc. of the standard biuret solution, containing 13.33 mgm. of biuret, equivalent to 12.3 mgm. of protein, into a 10 cc. graduated cylinder. Add distilled water to 8 cc., add 1 cc. of 30 per cent sodium hydroxide, 0.25 cc. of 20 per cent copper sulfate solution, then dilute to 10 cc. with water. Mix thoroughly, let stand 10 minutes, centrifuge. Transfer the supernatant fluid to the colorimeter cup, and compare with the solution of urine protein, setting the depth of the standard column at 15 mm.

Calculation:

$$\text{Grams protein per liter urine} = \frac{15}{R} \times \frac{12.3}{\text{cc. urine used}}$$

$$= \frac{184.5}{R \times (\text{cc. urine used})}$$

R being the depth of the protein solution matching 15 mm. of the standard.

Precipitation of globulin: To 10 cc. of urine prepared as described above, add 10 cc. of 44 per cent sodium sulfate solution, mix well, and place in an incubator at 37°C. for 3 hours. Filter until a perfectly clear filtrate is obtained.

Albumin: With the filtrate from the sodium sulfate precipitation proceed as described under "total protein," performing the precipitation tentatively with a volume of filtrate equal to 4-fold that of the urine taken for total protein determination.

Calculation:

$$\text{Grams albumin per liter urine} = \frac{15}{R} \times \frac{12.3 \times 2}{\text{cc. filtrate used}}$$

$$= \frac{369}{R \times (\text{cc. filtrate used})}$$

Globulin: The globulin is estimated by difference.

(Total protein) − (albumin) = (globulin).

The maximum error of the method is about ± 1 per cent for total protein and for albumin.

Experiments on globulin precipitation

In order to determine the solubility of globulin in 22 per cent sodium sulfate, globulin was prepared from horse serum according to the

TABLE 1

Solubility of globulin in 22 per cent sodium sulfate solution

Solution number	Strength of globulin solution	Amount of protein in filtrate
	per cent	*per cent*
1	0 52	0 011
2	1 56	0 016

method described by Haslam (19). Two solutions were tested. Solution 1 contained 0.52 per cent globulin. Solution 2 contained 1.56 per cent globulin. To each was added an equal volume of 44 per cent sodium sulfate, so that the precipitating mixture, contained 22 per cent of sodium sulfate. The procedure was carried out as described in the method for urine. The filtrate was analyzed for nitrogen by Kjeldahl. The results are shown in table 1.

From the results one may estimate that the amount of actual globulin in solution in the filtrate is 0.009 per cent, 0.002 per cent of impurity (presumably albumin) being dissolved in the filtrate when the 0.52 per cent globulin solution was precipitated, and 0.007 per cent when the 1.56 per cent solution was precipitated. We have, however, not corrected our results for this solubility, as it is too small to affect their significance.

To test whether the precipitation of globulin by 22 per cent sodium sulfate at 37°C. is complete in 3 hours, comparative determinations were made, allowing the precipitating mixture to stand 3 hours and 24 hours. Agreement was good, as is shown in table 2.

TABLE 2
Effect of precipitation time at 37°C. on globulin estimation

Determination number	Amount of globulin per liter	
	Incubation time	
	3 hours	24 hours
	grams	*grams*
1	3 8	3 6
2	2 6	2 6
3	2 4	2 4

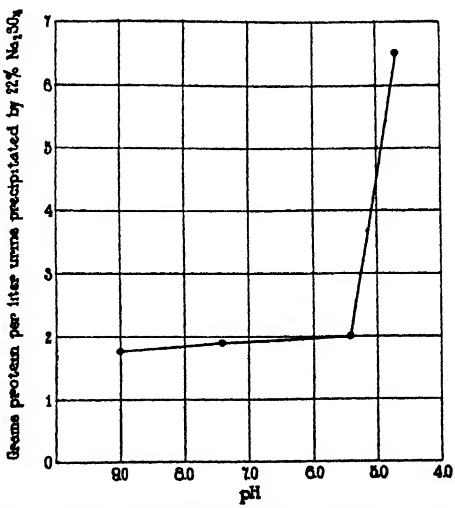

FIG. 1. THE EFFECT OF pH OF URINE ON THE PRECIPITATION OF GLOBULIN

In order to test the optimum reaction for the precipitation of globulin with sodium sulfate, portions of urine were adjusted to pH 9, 7.4, 5.4, the isoelectric point of serum globulin, and 4.7, the iso-

electric point of serum albumin. Figure 1 shows that between the isoelectric point of globulin and pH 9.0 the precipitation of protein is practically constant, while at the isoelectric point of albumin the amount of protein precipitated increases. For this reason the acid urines were adjusted to the alkaline side of neutrality.

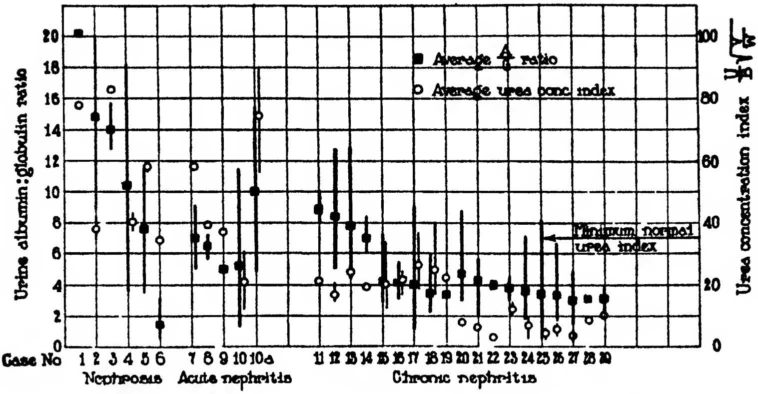

FIG. 2 SUMMARY OF CASES CLASSIFIED ACCORDING TO URINE ALBUMIN : GLOBULIN RATIOS AND UREA CONCENTRATION INDEX

For each patient a solid square indicates the average albumin globulin ratio during the observation period, the line running through the square indicates the extreme range of ratio found The open circle indicates the average urea concentration index, $\frac{U}{B}\sqrt{\frac{V}{W}}$, for a patient; the line running through the circle indicates the range of fluctuation of the index during the period of observation.

III. OBSERVATIONS IN NEPHRITIS

In a series of nephritic cases the *urinary proteins* were estimated by the above method. The *plasma proteins* were estimated by the method of Howe (17). *Blood urea nitrogen* was estimated by the method of Van Slyke and Cullen (20). *Blood creatinine* was estimated by the method of Folin and Wu (21).

The urea concentration index was calculated from the urea content of blood and urine by a modification of the original method of Austin, Stillman, and Van Slyke. The present index is calculated as $\frac{U}{B}\sqrt{\frac{V}{W}}$

and has been discussed in a previous publication (22). U = urine urea concentration. B = blood urea concentration V = urine volume output in cc. per hour. W = body weight. The index represents *the number of times the blood urea is concentrated in the urine when* $\frac{V}{W} = 1$, *or the volume output is 1 cc. per hour per kilo* (e.g., 60 cc. per hour for a 60 kilo person), which is the average normal output. When $\frac{V}{W} = 1$ the simple concentration ratio $\frac{U}{B}$ represents

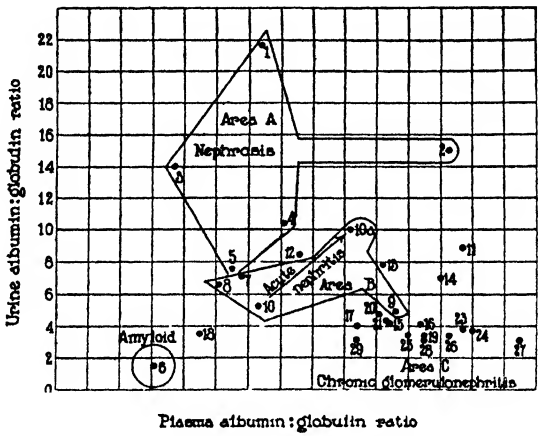

FIG. 3. RELATION OF THE AVERAGE ALBUMIN-GLOBULIN RATIO OF URINE TO THAT OF BLOOD PLASMA

The numbers are those of the cases shown in table 4. The arrow between 10 and 10a indicates the direction of change in case 10 during recovery.

the concentration index. With a urine of greater volume, U is naturally less, and is multiplied by the empirical volume factor $\sqrt{\frac{V}{W}}$ in order to bring it up to the value it would have when $\frac{V}{W} = 1$.

The results are shown in tables 3, 4, and 5, and figures 2 and 3. The types of nephritis are classified according to Volhard and Fahr as outlined by Linder, Lundsgaard, and Van Slyke (15).

The cases of nephrosis (tables 3 and 4 and figure 2) with one ex-

ception had high urine albumin: globulin ratios, mostly above 10. The urea concentration index was normal or high in all these cases, and the blood urea nitrogen was below 0.20 gram per liter.

Case no. 6, figures 2 and 3 and table 4, with a urinary albumin : globulin ratio of only 1.5 was not a pure nephrosis, but was complicated with pulmonary tuberculosis and amyloidosis. It has been pointed out by other authors (6, 7, 23) that *amyloid degeneration* is associated with a high output of globulin, resulting in a low albumin : globulin ratio in the urine. Csatáry (6) reported ratios below 1. Joachim (7) reported a ratio of 1.4. Gross (10) found ratios varying from 0.5 to 5. Our results showed a constantly low urinary albumin : globulin ratio, ranging from 0.5 to 3.1, and averaging 1.5.

TABLE 3

The albumin : globulin ratio in the urine in different types of nephritis

Classification	Number of cases examined	Number having A G ratio above 10	Number having A G ratio between 5 and 10	Number having A G ratio below 5	Number having blood urea nitrogen below 0 20 gram per liter	Number having blood urea nitrogen above 0 20 gram per liter
Nephrosis	6	4	1	1*	6	0
Nephritis, acute	4	0	4	0	3	1
Nephritis, chronic	19	0	4	15	5	14

* Complicated with pulmonary tuberculosis and amyloidosis. See discussion.

The four cases of *acute nephritis* (tables 3 and 4 and figure 2) had urine albumin : globulin ratios between 5 and 10. Only one case, no. 10, was observed during the whole course of the disease, and the results of this case can be distinctly classed into two groups (tables 4 and 5 and figures 2 and 3). The early stage of the disease was characterized by an average urinary albumin : globulin ratio of 5, and an average urea concentration index of 21. The period of recovery (10a, table 4, and figures 2 and 3) showed an average ratio of 10 and an average urea concentration index of 74. The blood urea nitrogen was at all times below 0.20 gram per liter. The remaining cases observed had normal urea concentration indices and blood urea.

Of the 19 cases of *chronic nephritis* observed, 4 had urinary albumin : globulin ratios between 5 and 10, while 15 had ratios below 5 (tables 3 and 4, and figure 2). The ratios roughly paralleled the

TABLE 4.—

Case number	Initials	Diagnosis	Age	Plasma proteins			Urine proteins average figures per 24 hours				Average urea concentration index $\frac{U}{B}\sqrt{\frac{V}{W}}$
				Albumin	Globulin	A.G	Albumin	Globulin	A.G ratio	Number of estimations	
			years	*per cent*	*per cent*		*grams*	*grams*			
1	W. J.	Nephrosis	27	1 65	2.21	0 75	15.1	0 7	21.6	2	78.0
2	G. G.	Nephrosis	24	2 40	2 04	1.18	7 5	0 5	14.9	3	37 8
3	B. S.	Nephrosis	12	1 02	2 75	0.37	4.2	0 3	14 0	4	82 6
4	M. R.	Nephrosis	29	1 83	2 50	0 73	13 5	1.3	10 4	6	39.8
5	B. B.	Nephrosis	23	1 46	2 47	0 59	9.9	1 3	7 6	8	58.0
6	G. D.	Nephrosis, amyloidosis, acute pulmonary tuberculosis	57	1 15	3 37	0 34	8 4	5 6	1.5	17	34.2
7	A. C.	Nephritis, acute	27	1.55	2.68	0 58	6.4	0.9	7.1	2	57.6
8	J. My.	Nephritis, acute	24	1 38	2 72	0 51	11 2	1.7	6 6	3	39.4
9	B. Bl.	Nephritis, acute	34	2 98	2 82	1 06	2 9	0 6	4.8	2	37.3
10	D. G.	Nephritis, acute	30	1 54	2 55	0 60	12 8	2.5	5 2	8	20.7
10a	D. G.	Nephritis, acute	30	2 45	2 75	0 89	7 0	0 7	10 0	6	73.7
11	R. V.	Nephritis, chronic	24	2 08	1 64	1 27	15 1	1 7	8 9	3	21.0
12	P. L.	Nephritis, chronic	28	1 98	2 61	0 76	5 0	0 6	8 4	3	16 9
13	M. McC.	Nephritis, chronic	20	2 01	3 23	0 62	3 9	0 5	7 8	3	23 5
14	E. W.	Nephritis, chronic	25	2 59	2 13	1 20	3 5	0 5	7 0	3	18 8
15	J. L.	Nephritis, chronic	16	2.05	1.96	1 04	6 7	1.6	4 2	9	20 2
16	R. N.	Nephritis, chronic	37	2 07	1 82	1 14	8.2	2 0	4 1	4	21.1
17	B. F.	Nephritis, chronic	24	1.77	2 08	0.85	3 2	0 8	4 0	8	26 3
18	S. J.	Nephritis, chronic	34	1 90	3 64	0 52	2 8	0 8	3 5	5	24.7
19	M. G.	Nephritis, chronic	12	2.85	2 57	1 03	1.4	0 4	3.5	2	22 2
20	E. L.	Nephritis, chronic	33	2 96	2 92	1.01	4.7	1 0	4 7	6	7.4
21	E. Rt.	Nephritis, chronic	46	2 65	2 57	1 03	5 0	1 2	4.2	5	6.1
22	V. S.	Nephritis, chronic	27	—	—	—	4 8	1 2	4 0	2	2 7
23	S. Ly.	Nephritis, chronic	15	2 33	2.54	0 92	8 1	2.1	3.9	7	12.0
24	J. C.	Nephritis, chronic	27	3 24	2 41	1.34	7 8	2.1	3 7	16	6 9
25	R. S.	Nephritis, chronic	31	2 59	2 39	1 08	9 8	2 9	3 4	17	4.1
26	C. A.	Nephritis, chronic	34	3.48	2.89	1 20	4 4	1 3	3.4	12	5 4
27	M. H. A.	Nephritis, chronic	20	3.09	2.31	1 34	5.0	1.6	3 1	7	3.4
28	H. L.	Nephritis, chronic	10	2.67	2 32	1 15	5 3	1.7	3 1	2	8.4
29	F. M.	Nephritis, chronic	13	2 62	2.79	0.94	3 4	1 1	3 1	4	10.0

* Values for the blood pressure and for the amount of edma and transudates are those found on admission, except in case 24, in which there was a long hospital stay before the observations were made. The findings have been recorded in this way in order to give as definite a picture as possible of the clinical condition, before it had been modified by treatment.

Summary of cases

Average blood urea nitrogen per liter	Average blood creatinine per 100 cc.	Blood pressure*	Peripheral edema*	Serous effu-sions*	Duration of life after last observation	Remarks
grams	*mgm.*					
0.082	1.36	118/68	++	+	–	Seen 13 months later; very edematous
0.124	1.60	126/70	+	0	–	No worse 15 months later
0.060	1.15	110/76	++	++	–	Perfectly well 3 years later
0.151	1 53	120/78	++	0	–	No worse 19 months later
0 081	–	108/70	++	++	–	No worse 18 months later
0 072	–	110/70	++++	++	4 days	
0 065	1 58	146/88	++	++	–	Still edematous 16 months later. Had lost ground
0 167	1.36	130/65	+	0	–	Not traced
0 233	1 57	160/92	+	++	–	Seemed recovered 15 months later
0 193	1 87	160/106	++	+++	–	Considerable improvement a year later
0 082	1 55	108/74	0	0	–	
0 139	1 59	148/76	+	0	–	Discharged unimproved 7 months later
0 280	1 84	164/90	+	0	–	Condition stationary 15 months later
0 153	1 62	166/98	++	0	9 months	Progress unfavorable. Died of diphtheria
0.218	2 01	150/92	++	±	6 months	Death probably from general septicemia
0 235	1 40	138/80	++	0	–	Seen 16 months later Kidney function worse but clinically better
0 180	1 64	138/92	++	+	17 months	Death from uremia and heart failure. Autopsy
0 124	1 37	100/70	++	+	18 months	Death from uremia and asthenia
0 154	1 36	134/78	++	+++	11 months	Death from uremia and heart failure
0 346	1 31	120/60	+	0	–	Observed for 2 years. Progress unfavorable
0 369	6 10	270/120	++	+	1 month	Edema and hydrothorax relieved by digitalis. Death from cardiac decompensation
0 292	2 52	250/92	+++	±	7 months	Died in coma (from cerebral edema?)
1 548	24 73	206/126	0	0	3 days	Death from uremia
0 280	–	158/95	0	0	–	Free from symptoms 3 years later
1 700	16 60	190/130	+	0	21 days	Death from uremia
0 658	7 33	156/100	++	+	3 months	Death from uremia
0 733	5 20	225/114	Very slight	0	few weeks	
0 794	6 09	162/118	+++	++	2 months	Cardiac edema. Death from cardiac decompensation
0.440	–	120/90	+	+	6 months	Death from uremia
0.200	1 67	208/145	++	++	7 months	Died of pneumonia

When the amount of peripheral edema exceeded a slight pitting it has been recorded in degrees, on a scale of 4. The record for transudates is similarly represented. The symbol ± is used to record the fact of a slight amount of dullness in the flanks, or impairment of resonance at the lung bases, when the actual presence of fluid could not be definitely substantiated.

urea concentration indices in these cases, and the majority of cases with ratios under 5 had urea concentration indices under 15.

TABLE 5

Case 10, acute nephritis, showing increase in albumin : globulin ratio during period of recovery

Date	Urine				Blood plasma				Remarks
	Total protein excreted per 24 hours	Albumin excreted per 24 hours	Globulin excreted per 24 hours	Albumin : globulin ratio	Total protein	Albumin	Globulin	Albumin : globulin ratio	
1925	*grams*	*grams*	*grams*		*per cent*	*per cent*	*per cent*		
January 11	16 1	13 8	2 3	6 0	4 70	1 59	3 11	0 51	Period of apparently stationary condition
January 12	16 4	15 1	2 4	6 3					
January 19	20.7	16 4	4 3	3 8	3 65	1 30	2 35	0 55	
January 26	18.5	15.8	2 7	5 9					
February 1	20 1	16 1	4 0	4 0					
February 12	23 3	14 1	2.3	6 1					
February 24	7.3	6 0	1.3	4 6	4 09	1 54	2.55	0 60	Profuse diuresis began, with loss of edema and with general improvement
February 25	6 4	5 3	1 1	4 8					
Average				5 2				0 55	
March 29	10 3	9 1	1 2	7 6	5 19	2.45	2 75	0 89	Period of recovery
March 31	9.7	9 0	0 7	12 9					
April 8	8 8	7 4	1.4	5 3	5 29	2 36	2 93	0.81	
April 15	7 7	6 9	0 8	8 6					
April 28	4 9	4 5	0 4	11 2					
April 29	7.7	7.2	0 5	14 4					
Average				10 0				0 85	

The *correlation between excretion of protein in the urine and loss of protein in the blood plasma* is shown in table 6 and figure 3. From the point of view of total protein lost, table 6 shows a general decrease

of total plasma protein with increase in the total protein excretion when the averages of protein excretion from a number of cases are considered. This finding is consistent with others in the literature, discussed previously.

In attempting to correlate the albumin : globulin ratio in the blood plasma with that in the urine, shown by figure 3, it can be said, in a general way, that the cases of nephrosis, area A, with high urine

TABLE 6

Relation of total protein in the urine to total protein in the blood plasma

Number of cases observed	Total protein		
	Plasma	Urine	
	Range	Range per 24 hours	Average per 24 hours
	per cent	*grams*	*grams*
1	6–7		5 3
11	5–6	1.6–12 0	5 8
10	4–5	1 3–16 1	8 6
7	3–4	3 3–16 3	9 8

TABLE 7

Relation between albumin : globulin ratio in urine and duration of life

	Total number of cases	Number still living	Number dead	Remarks
Cases with average A:G ratio exceeding 10	4	4	0	
Cases with average A:G ratio between 5 and 10	9	6	2	One case untraced
Cases with average A:G ratio under 5	16	3	13	Average duration of life in fatal cases 6 months

albumin : globulin ratios, tend to have lower plasma ratios, while the cases of chronic nephritis, area C, with low urine ratios tend to have the higher plasma ratios: the greater the proportion of albumin in the urinary protein loss the greater tends to be the albumin deficit in the blood plasma. The cases of acute nephritis, area B, fall irregularly between areas A and C. Case 10 shows the changes which occurred during recovery, from a low ratio in both urine and

plasma in the initial stages to higher ratios in the stage of recovery. In this case the above general rule is reversed; urinary and plasma albumin : globulin ratios showed a parallel instead of a reverse change. Case 6, with nephrosis and amyloidosis, is also an exception. The plasma and urine ratios are both lower than any others observed.

The general tendency towards an inverse relation between plasma and urine ratios would seem to point to excretion of one type of protein in the urine as at least a partial explanation for the loss of that protein in the plasma. In view of the deviations, however, it appears that other factors in addition to protein excretion are involved in the process of lowering the plasma proteins.

An attempt was made to correlate changes in the amount of protein excretion with changes in the volume of urine excreted, by recording daily excretions, 12-hour excretions, and hourly excretions. The results showed sometimes an increased protein excretion with increased volume, at other times no relationship, and, on a few occasions, the opposite effect.

The prognostic significance of the albumin : globulin ratio in the urine is shown in tables 4 and 7. Of the 4 cases with average ratios exceeding 10, one has recovered completely, while the remaining three show no signs of downward progress since the time of the original observations, which covers periods varying from 13 to 19 months (table 4).

Of the 9 cases whose average albumin : globulin ratio in the urine fell between 5 and 10, two (nos. 13 and 14) have died In both cases death was due to intercurrent infection, and cannot be ascribed to disease of the kidneys. None of the cases in the group have developed serious impairment of kidney function.

Of the 16 cases with average ratios under 5, 13 died within from 3 days to 18 months after the observations were made. One of these cases (no. 23) was seen a year later and had lost ground. He is still living and free from symptoms 3 years after the observation. The two remaining cases have been examined from time to time. Both of these (nos. 15 and 19) have shown a downward progress and kidney function is becoming progressively more impaired.

Each tabulated urinary albumin : globulin ratio is the average of [illegible] made on a number of different days. The range of

values for the ratios found during the period of observation, as well as the averages, are shown in figure 2. The variations from day to day in the ratios of some of the cases were fairly large.

This variation and the consequent overlapping shown in figure 2 are sufficiently great to invalidate conclusions from any single determination. The average of several days is required. The variations appear due to actual fluctuations in the proportions of albumin and globulin excreted, and not to errors in technique.

SUMMARY

The albumin and globulin in nephritic urines have been separated with Howe's sodium sulfate procedure, the separated proteins being determined by a modification of Autenrieth's colorimetric use of the biuret protein reaction. A satisfactory standard for this method has been found in pure biuret.

The albumin : globulin ratio of the urine proteins was found usually above 10 in nephrosis; between 5 and 10 in acute nephritis, with a low ratio during the early stage followed by a higher ratio during recovery; usually below 5 in the advanced stages of chronic glomerular nephritis with urea retention and impaired kidney function. In one case of amyloid nephrosis the ratio was very low, 1.5, in accord with the previous literature.

The low ratios in advanced chronic nephritis were associated with low urea concentration indices and poor prognoses. Of 15 cases observed with average urinary albumin : globulin ratios below 5, all but 2 have died in less than 18 months, and these 2 have shown progressive decrease in renal function.

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[Reprinted from The Journal of Experimental Medicine, June 1, 1927, Vol. xlv, No. 6, pp. 1081-1091.]

A FURTHER STUDY OF BLOOD REACTION AND BLOOD GASES IN PNEUMONIA.

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(Received for publication, February 28, 1927.)

Three years ago two of us with Neill and Morgan (1) attempted to determine whether or not a condition of acidosis exists in pneumonia. The hydrogen ion concentration, carbon dioxide content and tension, and the oxygen content and capacity were estimated in the blood of sixteen patients. In all, thirty observations were made. No tendency towards an acidosis of either metabolic or respiratory origin was observed. Indeed, the alkali reserve was within normal limits in every case. The pH, too, was in each instance within normal limits, 7.30 to 7.50, the greater number of observations falling in the more alkaline half of this range. The results, as are those of this study, were believed to contraindicate alkali therapy.

In spite of the consistency of the findings in this first series, it was thought advisable to extend the observations to a larger number of patients, and particularly to make repeated successive estimations on the blood of a few individuals. A patient suffering from pneumonia may present such a rapid succession of changes with respect to his hematorespiratory function, that he can scarcely be said to be in a steady state. What is true of him one day may not be true of him the next.

This fact, together with the changes induced by such environmental factors as serum therapy and oxygen inhalation, naturally gives one pause in generalizing about the state of the blood in so inconstant a condition as pneumonia. The results of the present study are, however, corroborative of the earlier one.

Material and Methods.

The observations reported were made on adult patients of both sexes, all suffering from pneumonia. Nineteen individuals comprise the series on whom

TABLE I.

Case No.	Observation No	History No	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO_2 content per liter	CO_2 tension	O_2 content per liter	O_2 capacity per liter	Saturation	Temperature	Pulse	Respiration	Breathing room air	Breathing O_2. O_2 in chamber	Treatment	Remarks
										mM	mm Hg	mM	mM	per cent	°C				per cent		
1	1	5230	2- 2-25, a m.	Type I	R. U. L.	6			7 30	21.52	52 4	6 02	8 18	73.6	101 4	118	40	Yes		Type I serum	Empyema Surgical drainage
	2		2- 2-25, p m.		R. U. L.	6			7 48	21 20	36 0	7.75	8 51	91.1	100 4	102	40		39	Type I serum	Suppurative pleurisy and pericarditis
	3		2- 5-25, a.m.		Empyema	9			7 45	25 80	44 8	6 74	6 93	97 3	100 4	98	40		38	Type I serum	Death
	4		2- 5-25, p m.		Empyema	9			7 50	25 22	39 1	5 73	6 95	82 5	101.4	96	36	Yes			
	5		2- 6-25		Empyema	10		42	7 47	24 52	40 7	5 95	7 01	84 9	101 4	104	36	Yes		Transferred for operation	
2	6	5233	2- 4-25	Grp. IV	R. U. L.	3			7 53	22 03	33 1	7 59	7 80	97 3	100 4	125	24	Yes			Recovery
	7		2- 6-25		R. U. L.	5	6		7 38	24 92	51 9	7 30	8 51	85 9	100 4	88	32	Yes			
3	8	5253	2-20-25	Type I	R. U. L.	3		4	7 42	22 45	45 3	8 13	9 36	86 8	101	120	32		38	Type I serum	Bilateral sero-fibrinous pleurisy. Death

4	9	5257	2-21-25, a m.	Grp IV Staph.	R L. L.	2			7 47	22 38		6 65		68 3	100 4	120	40	Yes				Bilateral sero-fibrinous pleurisy. Fibrinous pericarditis. Death
	10		p.m.		R L. L.	2		3	7 50	22 56	37 6	7 42	9 69	76 6	100 4	130	36		38			
5	11	5259	2-23-25	Grp. IV	L. L. L.	2			7 40	20 55	41 3	5 73	8 04	71 3	100 4	116	38	Yes				Death
	12		2-24-25		L. L. L.	3		3	7 45	19 95	36 3	6 44	8 51	75 7	103 4	130	36	Yes				
6	13	5268	3- 2-25	Grp IV	R. U L.	4			7 46	22 96	38 9	5 73	6 82	84 0	102.1	76	31	Yes				Recovery
	14		3- 3-25			5			7 49	24 20	38 3	6 01	6 53	92 0	101 6	72	30		39			
	15		3- 4-25			6			7 49	24 86	39 1	6 15	6 32	97 3	101	80	34		41			
	16		3- 5-25			7	7		7 40	26 70	50 2	5 90	6 40	92 2	98 6	64	28		39			
	17		3- 6-25			8			7 48	25 60	40 1	5 70	6 01	94 9	97 6	60	22	Yes				
	18		3- 7-25			9			7 44	24 28	41 9	5 94	6 52	91 1	97 8	68	24	Yes				
	19		3- 9-25			11			7 34	19 69	42 1	6 31	6 58	95 9	98 4	68	22	Yes				
	20		3-10-25			12			7 45	20 27	34 8	6 32	6 86	92 2	98 6	60	25	Yes				
	21		3-13-25			15			7 37	21 74	43 6	6 40	6 64	96 4	98 2	76	20	Yes				
7	22	5278	3- 8-25	Grp IV	R. L. L.	4			7 41	23 08	43 9	6 31	6 93	91 1	103	108	28	Yes				Recovery
	23		3-10-25			6			7 44	23 31	41 4	6 28	7 16	87 7	101 3	108	24	Yes				
	24		3-11-25			7			7 46	23.58	41 7	6 29	6 71	93 8	100 4	88	32	Yes				
8	25	5292	3-19-25	Grp IV	L. L. L.	3	11		7 40	20.59	41 5	7 56	7 95	95 1	103 6	104	28	Yes				Recovery
9	26	5300	3-23-25	Type I	L. L. L. R. U. L.	4			7 37	25 60	53.4	4 92	7 67	64 2	102	110	48	Yes			Type I anti-pneumococcus serum	Empyema. Surgical drainage. Recovery
	27		3-24-25			5			7 33	26 82	60 9	7 15	7 63	93 7	101	92	36		40			
	28		3-25-25			6	9		7 43	23 73	43 7	6 68	7 83	85 4	100 4	72	34	Yes				

ABLE I—*Concluded.*

Case No.	Observation No	History No	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO_2 content per liter	CO_2 tension	O_2 content per liter	O_2 capacity per liter	Saturation	Temperature	Pulse	Respiration	Breathing room air	Breathing O_2. O_2 in chamber	Treatment	Remarks
										mM	*mm Hg*	*mM*	*mM*	*per cent*	°C				*per cent*		
10	29	5310	3–27–25	Type I	R. M. L. R. L. L.	5			7 38	24 30	52 7	7.56	9 76	77 5	103 2	106	32	Yes		Type I anti-pneumococcus serum	Death
	30		3–28–25			6			7 49	23 41	39 8	7 69	8 84	87 0	104	118	50		39		
	31		3–31–25			9		10	7 42	29 61	52 1	3 55	4 98	71 3	101 8	138	56		38		
11	32	5314	3–30–25, a.m.	Type III	L L. L.	7			7 33	20 70	44 8	3 55	5 68	62 5	102 8	86	32	Yes			Recovery
	33		3–30–25, p.m.				13		7 49	21 80	33 3	4 26	5 33	80 0	102	84	50		50		
12	34	5317	4– 6–25	*Staph. aureus*	R L L. R. M. L.	7			7.50	21 55	32 1	4 88	5 37	90 9	101 2	100	28		40		Empyema Surgical drainage. Recovery
13	35	5347	5–18–25, a.m.	Type III	L U. L. L. L. L.	7			7.34	21 95	50 1	6 47	9 07	71 3	100 6	124	32	Yes			Septicemia (Type III). Chronic morphine poisoning. Recovery
	36		5–18–25, p.m.			7				23 55		8 80	8 97	98 1	98 2	112	32		50		
	37		5–23–25			12			7 48	27 40	45 5	6 19	8 02	77 2	99 2	104	36	Yes			
	38		5–25–25			14			7 41	28 44	54 2	7 20	7 64	94 3	98 8	76	22		40		
	39		6– 2–25			15				27 20		5 24	7 96	65 8	100 4	90	28	Yes			
	40		5–26–25			22	7		7 44	32.55	6 25	5 53	6 43	86 0	98 6	73	16	Yes			

14	41	5470	12–15–25	Type III	L. L. L.	4	5		7 47	25 88	41.8	5.54	5.74	96.5	102.2	100	32	Yes			Recovery
15	42	5472	12–21–25	Grp. IV	R. U. L. R. L. L.	7	12		7 38	25 76	48 6	2 74	4 41	62 1	102	120	42	Yes			Recovery
16	43	5476	12–19–25	?	L. L. L.	5	11		7 37			4 99	5.22	95.6	104	72	25	Yes			Recovery
17	44	5490	12–30–25, a.m.	Type I	L. U. L. L. L. L.	4			7 50	32 62	49 1	3 02	5 48	55.1	102	130	38	Yes		Type I serum. Transferred for operation	Empyema. Surgical drainage. Recovery
	45		12–30–25, p.m.				12		7 46			4 65	5 29	87 9	102 3	116	32		45		
18	46	5509	1–12–26	Grp. IV	L. U. L. L. L. L.	3		7	7 40	22.58	43 9	6 13	7 18	85 4	102 4	116	30	Yes			Confluent bronchopneumonia. Death
19	47	5522	2– 1–26	Grp. IV	R. U. L. R. M. L. R. L. L.	12		13	7.36	32 22	65 6	3 70	6.32	58 6	99 4	120	70		40		Death

R. U. L. signifies right upper lobe; R. M L., right middle lobe, R. L. L., right lower lobe, L. U. L , left upper lobe; L. L. L., left lower lobe.

forty-seven observations were made. The number of observations per individual varied from one to as many as nine. Blood for analysis was drawn by puncture, usually of the femoral artery. The customary precautions were taken against exposure of the blood to air. Analysis of the total carbon dioxide content, oxygen content and capacity, was performed by the method of Van Slyke and Neill (2). The hydrogen ion concentration was measured by the colorimetric method of Hastings and Sendroy (3).

Calculations.

The data presented in Table I and graphically plotted in Fig. 1 were derived, as in the first paper, by calculation from the analyses of

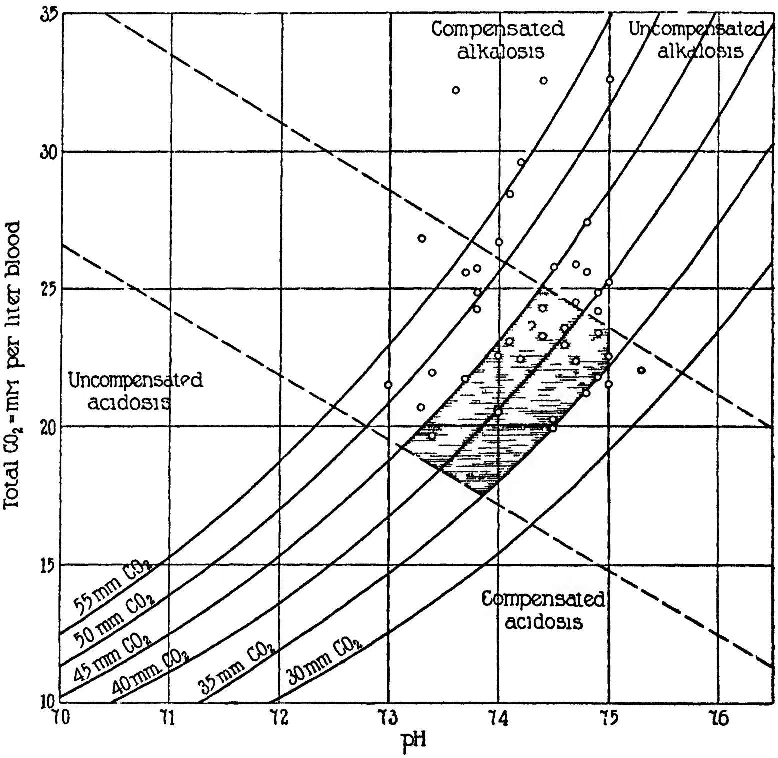

FIG. 1.

pH of serum, and CO_2 content, oxygen content, and oxygen capacity of whole arterial blood.

The fundamental equation used was the familiar Henderson-Hasselbalch formula for whole blood:

$$(1) \qquad pH_{t^\circ} = pK_1{}'_{bt^\circ} + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

$$= pK_1{}'_{bt^\circ} + \log \frac{[CO_2] - [H_2CO_3]}{[H_2CO_3]}$$

where t° = body temperature in °C., $pK_1{}'_{bt^\circ}$ = the negative logarithm of the apparent first dissociation constant of carbonic acid in whole blood at body temperature, and the brackets indicate concentrations in terms of mM per liter whole blood.

$$(2) \qquad [H_2CO_3] \qquad \frac{[CO_2]}{1 + 10^{\,pH - pK_1{}'_{bt^\circ}}}$$

The pH of serum, as usual, was used for whole blood. The value of $pK_1{}'_b$ was calculated from the equation of Van Slyke, Wu, and McLean (4).

$$(3) \qquad pK_1{}'_b = pK_1{}'_s + \Delta\, pK_1{}'$$

where $pK_1{}'_s$ stands for $pK_1{}'$ in serum, and $\Delta pK_1{}'$ is the correction applied for the difference in $pK_1{}'$ in whole blood from the serum value. From the pH, oxygen content, and oxygen saturation, $\Delta pK_1{}'$ was estimated by using the nomogram of Van Slyke, Hastings, Murray, and Sendroy (5). To estimate $pK_1{}'_{bt^\circ}$, the data of Stadie and Martin (6) were used to calculate the temperature coefficient of this constant. Their values give $\frac{\Delta pK_1{}'_b}{\Delta t^\circ} = -0.0065$. Hence the complete expression for $pK_1{}'_{bt^\circ}$ is

$$(4) \qquad pK_1{}'_{bt^\circ} = pK_1{}'_{s38^\circ} + \Delta pK_1{}' \pm \Delta t^\circ\,(-\,0.0065)$$

where $pK_1{}'_s$ = 6.12, and $\pm\, t^\circ$ is the difference in °C. between body temperature at the time of bleeding and 38°C.

To estimate the CO_2 tension, the expression:

$$(5) \qquad [H_2CO_3] = \frac{\alpha CO_{2_{bt^\circ}} \times pCO_2}{760 \times 0.0224} = 0.0587\alpha CO_{2_{bt^\circ}} \times pCO_2$$

was used, where αCO_{2bt° is the solubility coefficient of CO_2 in whole blood at body temperature, and pCO_2 is the CO_2 tension. The value of αCO_{2bt° being propor-

tional to the water content of the blood. this quantity was calculated as follows. Using equation 30 of Van Slyke, Wu, and McLean (4),

$$(6) \qquad (H_2O)_b = 0.914 - 0.015\ (Hb)$$

where $(H_2O)_b$ = kilos H_2O per kilo blood, and Hb = mM Hb per kilo blood as measured by oxygen capacity determinations, the expression:

$$(7) \qquad H_2O_b = \frac{0.914\ (Gb) - 0.015\ Hb}{1.007}$$

was derived, where H_2O_b = liters H_2O per liter blood, (Gb) = specific gravity of blood, and Hb = mM per liter blood. (Gb) was calculated from equation 29 of Van Slyke *et al.*:

$$8) \qquad (Gb) = 1.027 - 0.0037\ Hb$$

According to Bohr and Bock, $\alpha CO_{2_{H_2O}}$ at 38° = 0.555 and the temperature coefficient at this temperature is −0.0124.

$$(9) \qquad \text{Hence } \alpha CO_{2_{bt^\circ}} = [0.555 \pm \Delta t^\circ\ (-\ 0.0124)]\ H_2O_b$$

By combining all of the previous equations, the complete expression for the CO_2 tension is found to be:

$$(10)\ pCO_2 = \frac{[CO_2]}{0.0583 \times [0.555 \pm \Delta t^\circ\ (-\ 0.0124)] \times [0.939 - 0.01162\ Hb] \times \left[1 + 10^{pH - [6.12 + \Delta pK_1' = \Delta t^\circ (-\ 0.0065)]}\right]}$$

Due to the fact that the average Hb concentration and degree of saturation was different from that of the group of cases reported in the first paper, the results, when plotted on the diagram of Hastings, Neill, Morgan, and Binger (1), showed a CO_2 tension different from that calculated. Hence a new diagram was drawn based on calculations of a blood having an average oxygen capacity of 7 mM Hb per liter, and an oxygen saturation of 82 per cent. Calculated CO_2 tensions in most instances agree with those plotted in Fig. 1.

RESULTS.

The results are tabulated in Tables I and II and graphically plotted in Fig. 1, on the modified Van Slyke acid-base diagram.

The pH of the Serum.—Of the 45 observations but one pH was higher than 7.50, all the rest falling within the normal limits of 7.30 to 7.50. The average pH of this series is 7.43. Table II shows a higher frequency of pH on the alkaline side of this normal range.

TABLE II.

Showing the Frequency of Incidence of the Variables Calculated in Table I.

pH	7 30–35	7.36–40	7 41–45	7 46–50	7.51–55			
Incidence	5	11	11	17	1			
CO_2, *mM per liter*	19–21	21.01–22 0	22 01–24 0	24 01–26 0	26 01–28 0	28 01–30.0	30.01–32.0	32.01–34 0
Incidence	5	5	12	14	4	2	0	3
pCO_2, *mm*	30–35	35 1–40 0	40 1–45 0	45.1–50 0	50 1–55 0	55.1–60.0	60.1–65.0	65.1–70.0
Incidence	4	8	14	4	9	1	1	1
O_2 saturation, *per cent*	75 and below	76–80	81–85	86–90	91–95	96–100		
Incidence	12	4	5	7	13	6		

The CO_2 Content of the Blood.—The average of 42 measurements gave a value of 24.26 millimols per liter, which is well within the normal range. Most of our results were clustered about this figure for CO_2 content.

The CO_2 Tension.—Of the 42 CO_2 tensions calculated, half were within the normal limits of 35 to 45 mm., most of the remainder occurring above the higher of these values. The average was 44.4 mm.

The Oxygen Saturation.—The average value for oxygen content was 6.02 mM per liter, and for oxygen capacity 7.12 mM per liter. The average percentage saturation was 84.5 per cent, irrespective of oxygen therapy.

These results, in so far as one may make any inferences under such varying conditions of the disease and its treatment, corroborate, in every respect but one, the observations of the previous paper on this subject. This series fails to show the correlation previously noted, between the CO_2 tension and the patient's temperature. None of the points plotted in Fig. 1 fall in an acidosis area. In fact, those points not in the normal area tend to indicate a condition of compensated alkalosis. The CO_2 content showed little departure from normal values, as has already been noted by various other investigators. The CO_2 tensions, on the other hand, were grouped more in the region of the higher normal values. The oxygen unsaturation in these observations was more marked than that of the first series.

SUMMARY AND CONCLUSIONS.

Estimation of the blood reaction and of blood gases in a series of nineteen individuals suffering from pneumonia failed to reveal a condition of acidosis occurring at any time during the disease. The results of this study are corroborative of a previous one, to which reference has been made.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 1065–1080.]

THE RESPONSE TO RESPIRATORY RESISTANCE.

A COMPARISON OF THE EFFECTS PRODUCED BY PARTIAL OBSTRUCTION IN THE INSPIRATORY AND EXPIRATORY PHASES OF RESPIRATION.

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(Received for publication, February 28, 1927.)

The liability to fatigue of the respiratory center is a subject which needs to be studied. Davies, Haldane, and Priestley (1) were the first to investigate the manner in which breathing adapts itself to increased resistance, and the point at which the adaptation begins to fail. They showed that the normal response in man to respiratory resistance in both phases of respiration is slow and deep breathing. When the resistance is excessive respirations become progressively shallower and more frequent and the subjects then become cyanotic. Haldane and his coworkers believe that this is due to fatigue of the respiratory center. They believe anoxemia hastens greatly the onset of fatigue and the ease with which it is produced. They conclude that the mechanism involved in the immediate response is the Hering-Breuer reflex, pointing out that as a result of resistance, the time required for inflation or deflation of the lungs to reach the point at which the Hering-Breuer stimulus becomes effective is prolonged, that CO_2 accumulates in the meantime, and that the next respiration is deep and vigorous. The more or less sudden onset of rapid, shallow breathing Haldane interprets as evidence of fatigue of the respiratory center, with a resulting predominance of the peripheral stimuli over the central impulses normally governing breathing.

A study of these effects in animals was undertaken by us with several points in mind. We hoped for additional information as to the nature and origin of rapid and shallow breathing, which we have previously considered in both clinical and experimental studies (2–5). It seemed highly desirable to learn something about the liability to fatigue of so vital a structure as the respiratory center. Transferring the problem

to experimental animals rather than man, though it introduced such complications as the use of anesthetics, afforded the opportunity of allowing the experiments to go to their natural conclusion. It made it possible, too, to study the end-results of more or less prolonged periods of anoxemia and rapid and shallow breathing. It was soon learned that the response to resistance in inspiration is strikingly different from the response to resistance in expiration, both as regards functional and structural changes.

Method.

Dogs anesthetized with barbital-sodium were used. The dogs varied in weight from 6.8 to 23.5 kilos, but in every instance except one the weight of the animal was above 10 kilos. The barbital-sodium was dissolved in physiological sodium chloride solution and given intravenously in an amount sufficient to produce complete relaxation and a slow, steady respiratory rate The initial dose was calculated on a basis of 0.3 gm drug to 1 kilo body weight and the additional drug was given in repeated small quantities as necessary.[1] The rectal temperature was recorded at frequent intervals throughout the course of each experiment As a precaution against the loss of heat each animal was wrapped snugly in woolen blankets and surrounded by warm air [2]

When the animal had reached the desired state of anesthesia, tracheotomy was done, and a properly fitting rubber tube was tied firmly into the trachea This tube, which was of such a length as not to increase the dog's natural dead space, communicated with one arm of a four-way metal tube. Two of the other branches of the four-way tube were connected by corrugated tubing of the usual type used in respiration experiments to inspiratory and expiratory valves. Low resistance valves of the kind recently described by Dr. C. V Bailey[3] (6) were used in all experiments

The fourth opening in the metal tube was connected to a rubber tambour by means of a short length of rubber pressure tubing. This tambour, moving with expiration and inspiration, activated a make and break contact in an electrical circuit which included an electromagnet "telephone" counter. By this arrangement the respiratory rate was automatically counted Intratracheal pressure was measured by a water manometer communicating by means of a Y-tube with the

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[1] A 5 per cent solution was used for this purpose.

[2] A cradle made by covering an arch of thin metal with hair felt was placed over the animal. Heat was supplied by an electric bulb suspended from the top of the arch. We have found that this is a much more efficacious way of maintaining the warmth of an animal than by use of an electric pad.

[3] These valves were kindly supplied us by Dr. Bailey.

four-way tracheal tube. The expired air was collected in a large Tissot spirometer.
To introduce resistances we used a specially constructed metal tap with an

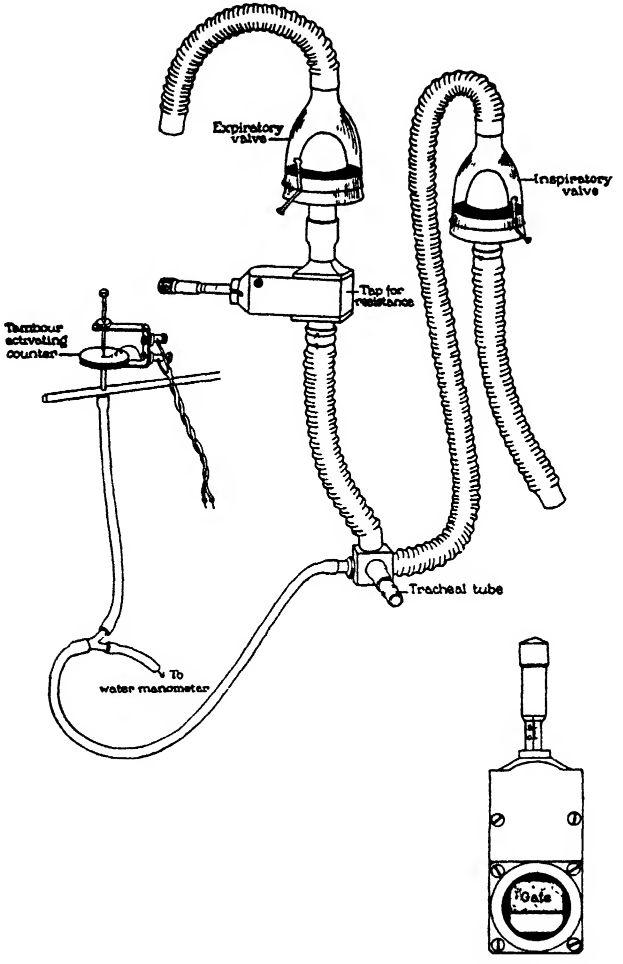

FIG. 1.

internal bore of 4 sq. cm. The tap was closed by means of a gate which was gradually lowered into its seat by a screw of such a pitch that one turn lowered the gate 1 mm. and accordingly diminished the lumen by 0.2 sq. cm. A scale permitted one

to read off at any time the cross-section area in sq. cm. This tap was introduced into the system at a point between the trachea and one of the respiratory valves. According to its position, resistance could be created either in the inspiratory or expiratory phases of respiration. The apparatus which we used is shown in diagram in Fig. 1.

When resistance to respiration is introduced by a tap of this sort the velocity of air flow varies as the square root of the driving pressure. With cotton wool resistance, however, which Haldane and his coworkers used, the air flow varies directly as the driving pressure. The intratracheal pressure may be assumed to represent the driving pressure. Velocity of air flow can thus be calculated if desired. We have chosen tap resistance because it is easier of manipulation and quantitative control. It was found that great reduction in the cross-section area of the tap was necessary before any change in type of breathing occurred.

All experiments were conducted on the same general plan and the observations fall into three periods; first, the period of control; second, the period of resistance; and, third, the period following release.

The observations include a record of respiratory rate, tidal air, minute volume of pulmonary ventilation, intratracheal pressure, cross-section area of the tap, and in some instances an additional study of the O_2 content, O_2 capacity, percentage oxygen saturation, CO_2 content, CO_2 tension, and pH of the arterial blood. The blood samples were withdrawn through a cannula in the femoral artery and collected without exposure to air in sampling tubes over mercury. The analyses of oxygen content and capacity, and of CO_2 content of the separated plasma or serum were made by the method of Van Slyke and Neill (7). The pH was estimated on serum by the colorimetric method of Hastings and Sendroy (8). From these data and the following formula (9) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pK′ to be 6.115.

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter.

The records of respiratory rate and pulmonary ventilation were made over 5 minute periods, and were repeated several times under each condition. During the time of resistance the cross-section area of the tap was reduced to a degree that created a negative or positive intratracheal pressure of from 10 to 20 cm. of H_2O, and this was maintained over intervals ranging from 20 to 145 minutes. The readings in the final period were taken when the respiratory rate had reached a constant, or approximately constant, level.

In some experiments the animals breathed room air. In others they breathed 90 to 95 per cent oxygen from a Douglas bag. After the final observations the experiment was terminated by the intravenous injection of from 20 to 30 cc. of a saturated solution of magnesium sulfate. At autopsy attention was given to the presence or absence of froth in the trachea, pleural effusions and gross edema of

the mediastinal tissues and lungs, and to the color of the lungs. The degree of hypostatic congestion, the lung weights, the heart weights, and the lung-heart ratio were also observed.

TABLE I.

Experiment 7. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc	liters	mM	mm. Hg		mM	mM	per cent
Control period	11 57 to 12 46	Room air	19	273	5.11	25 8	32 91	7 50	8 47	9 02	93 9
During resistance	12 46 to 2 55	Room air	30	92	2 78	28 1	53 15	7 32	6 29	9.39	67 0
After release	2 55 to 3 48	Room air	40	225	9 00	24 5	28 63	7 54	8 89	9 66	92.0

Weight of animal, 23.5 kilos.
Total barbital-sodium, 0.35 gm. per kilo body weight
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 20 cm. H_2O.
Duration of greatest resistance, 113 min.
Lung-heart ratio, 1.66.

EXPERIMENTS.

I. Experiments with Resistance in the Inspiratory Phase.

Five experiments were performed in which the cross-section of the tap opening was 0.05 sq. cm. In these experiments the negative intratracheal pressure during the inspiratory phase varied from 11 to 20 cm. of H_2O. In a sixth experiment a conspicuous effect on breathing was obtained with a cross-section area of 0.1 sq. cm. All animals showed an increase in respiratory rate and a decrease in tidal air. The percentage increase in respiratory rate ranged from a low extreme of 58 per cent to a high extreme of 310 per cent. and the percentage

decrease in tidal air ranged from a high extreme of 88.9 per cent to a low extreme of 56.1 per cent. The experimental data are presented in Tables I to IV.

Examination of Table I will bring out the following points. A dog anesthetized with barbital-sodium was breathing at the rate of 19 per

TABLE II.

Experiment 1A. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood: O_2 content	Arterial blood: O_2 capacity	Arterial blood: Saturation
				cc	*liters*	*mM*	*mm Hg*		*mM*	*mM*	*per cent*
Control period	12 47 to 1 35	90–95 per cent O_2	15	189	2 73	26 29	41 8	7 40	9 46	8 77	107 8
During resistance	1 35 to 3 04	90–95 per cent O_2	47	21	1 00	32 08	80 2	7 19	5.27	10 44	50 4
After release	3 04 to 3 24	90–95 per cent O_2	139	75	10 4	25 61	49 7	7 31	5 75	10 66	54 0

Weight of animal, 14 kilos.
Total barbital-sodium, 0.37 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Negative intratracheal pressure not recorded.
Duration of greatest resistance, 19 min.
Lung-heart ratio, not determined.

minute. The caliber of the tap was gradually reduced until it had reached 0.05 sq. cm. This was sufficient to cause a negative intratracheal pressure at the height of inspiration equal to 20 cm. H_2O. Under these conditions the rate accelerated to 30. This was accompanied by a decrease in tidal air from 273 cc. to 92 cc. and a resulting

decrease in minute volume of pulmonary ventilation from 5.11 liters to 2.78 liters. These changes occurred while the animal was breathing room air. They were associated with a rise in pCO_2 from 32.91 mm. to 53.15 mm., a drop in pH from 7.50 to 7.32, and a decrease in the percentage oxygen saturation of the arterial blood from 93.9 to 67.0. Resistance was maintained for 113 minutes. 65 minutes after the

TABLE III.

Experiment 5. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc	*liters*	*mM*	*mm Hg*		*mM*	*mM*	*per cent*
Control period	12 35 to 1 31	Room air	8	249	1 98	25 26	52 05	7 28	9 37	10 34	90 6
During resistance	1 31 to 3 49	Room air	17	80	1 36	29 40	61 95	7 27	6 00	10 48	57 3
After resistance	3 49 to 4 07	Room air	8	285	2 28	25 75	53 05	7 28	9 17	10 01	91 6

Weight of animal, 17 25 kilos.
Total barbital-sodium, 0 32 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq cm.
Negative intratracheal pressure, 11.5 cm. H_2O.
Duration of greatest resistance, 124 min
Lung-heart ratio, 1.71.

valve was opened the respiratory rate was 40, the tidal air was 225 cc., and the minute volume was 9 liters. The pCO_2 had dropped to 28.63 mm., the pH had risen to 7.54, and the percentage oxygen saturation of the arterial blood was again normal, 92 per cent. Postmortem examination showed no froth in the trachea, no effusion into either pleural cavity, and no edema of the mediastinal tissues. The surface of the lungs appeared granular; the color was a bright pink. Hy-

postasis was limited to the dependent parts of the lobes. The lung-heart ratio was 1.66.

In Experiment 1A (Table II) the animal breathed 90 to 95 per cent oxygen. In spite of this, after inspiring against resistance for 21 minutes the percentage oxygen saturation of the arterial blood dropped from 107.8 to 50.4. The rate accelerated from 15 to 47 and the tidal air dropped from 189 cc. to 21 cc. The tension of CO_2 rose from 41.8 mm. to 80.5 mm. and the pH decreased from 7.40 to 7.19. The final estimations in this animal were made 34 minutes after the resistance was removed. It should be noted that the respiratory rate at this time was 139, the tidal air only 75 cc., the tension of carbon dioxide still above the original level, and the percentage oxygen saturation of the arterial blood only 54. Autopsy showed a considerable amount of frothy liquid in the trachea and 10 to 15 cc. of clear fluid in each pleural cavity. The lungs were boggy with fluid.

In Experiment 5 (Table III) the increase in CO_2 tension during the period of resistance was slight, and the pH did not change. The animal breathed air, and the percentage oxygen saturation of the arterial blood dropped from 90.6 to 57.3. This accompanied an increase in respiratory rate from 8 to 17 and a decrease in tidal air from 249 cc. to 80 cc. Resistance was continued 124 minutes. 41 minutes after release all functions were at the normal level. Autopsy in this case showed no froth in the trachea, no effusion into either pleural cavity, and no edema of the mediastinal tissues. The lungs were diffusely coral-pink in color. Hypostatic congestion was slight. The lung-heart ratio was 1.7.

The data for Experiments 2, 3, and 4 are grouped together in Table IV. The results in general are consistent with those obtained in Experiments 1A and 7. The increase in minute volume during the period of resistance in Experiment 3 was a single occurrence. In Experiments 2 and 3 the animals breathed 90 to 95 per cent oxygen and maintained a pink color of the tongue and mucous membranes throughout. It seems safe to conclude that resistance in these animals did not produce anoxemia. At autopsy pleural effusions were found in Experiments 3 and 4. In Experiment 3 there was in addition an excessive edema of the mediastinal tissues. The color of the lungs in all cases was a coral-pink, of varying shades of intensity, but deeper in every instance

TABLE IV.

Experiments 2, 3, and 4.

The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Experiment 2					Experiment 3					Experiment 4				
	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume
				cc	*liters*				*cc*	*liters*				*cc*	*liters*
Control period	2 27 to 2 41	90–95 per cent O_2	18	114	2 09	11 01 to 11 22	90–95 per cent O_2	10	184	1 84	12 03 to 1 29	Room air	19	203	3.86
During resistance	2 41 to 5 15	90–95 per cent O_2	35	50	1 76	11 22 to 2 18	90–95 per cent O_2	41	64	2 62	1 29 to 4 30	Room air	39	80	3 24
After release	5 15 to 6 04	90–95 per cent O_2	44	90	3 98	2 18 to 3 40	90–95 per cent O_2	118	105	12 42	4 30 to 5 14	Room air	34	172	5 84

Experiment 2: Weight of animal, 6 8 kilos. Total barbital-sodium, 0 46 gm per kilo body weight. Cross-section area of tap finally reduced to 0 05 sq. cm. Negative intratracheal pressure, 14 5 cm H_2O. Duration of greatest resistance, 38 min. Lung-heart ratio, 1 75.

Experiment 3: Weight of animal, 17 kilos. Total barbital-sodium, 0 30 gm. per kilo body weight. Cross-section area of tap finally reduced to 0 05 sq cm. Negative intratracheal pressure, 15 5 cm. H_2O. Duration of greatest resistance, 145 min. Lung-heart ratio, 2 12.

Experiment 4: Weight of animal, 16 5 kilos. Total barbital-sodium, 0 33 gm per kilo body weight. Cross-section area of tap finally reduced to 0 05 sq. cm. Negative intratracheal pressure, 15 cm. H_2O. Duration of greatest resistance, 70 min. Lung-heart ratio, 1.53.

than the shade of a normal lung. Hypostatic congestion in Experiments 3 and 4 was moderate. The lungs in Experiment 2 were completely free of hypostasis.

The results of these experiments may be summed up as follows: Resistance to inspiration results in a fall in intratracheal pressure which is associated with an increase in respiratory rate and a decrease in tidal air. In most instances these are accompanied by a severe limitation of the minute volume of pulmonary ventilation. The effects come on suddenly when the cross-section area of the inspiratory passageway is reduced to 0.1 sq. cm. or 0.05 sq. cm. Anoxemia accompanies these changes, but may be prevented by the inhalation of 90 to 95 per cent oxygen. Associated with the anoxemia there is a retention of carbon dioxide and usually a drop in pH.

When resistance is removed the respiratory rate continues to be rapid. In the majority of instances the rates were higher following release than they were during the period of resistance. Release, however, permits an increase in tidal air and minute volume, and as a result of this, there is a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia. On only one occasion when resistance was removed did the respiratory rate return to the control level, with a corresponding change in CO_2 tension and oxygen saturation. The postmortem picture found in these dogs may be characterized by congestion and edema of the lungs

II. Experiments with Resistance in the Expiratory Phase.

In striking contrast to the experiments with resistance in the inspiratory phase, partial obstruction to expiration slows the respiratory rate. This occurs with varying effects on tidal air, but with a constant decrease in minute volume of pulmonary ventilation. The results of four experiments are presented in Tables V to VII.

In Experiment 11 (Table V) the dog breathed at the rate of 25 before resistance was introduced in expiration. In the presence of restriction equal to 0.1 sq. cm. the rate dropped to 12 and the tidal air increased from 85 cc. to 122 cc. This was accompanied by a decrease in minute volume from 2.12 liters to 1.46 liters with only slight changes in pH and blood gases. The animal exhaled against resistance for 96 minutes. 24 minutes after the tap was opened the

respiratory rate had returned to the original level—thus distinguishing this type of experiment from the ones in which resistance was introduced into inspiration. No other conspicuous changes occurred. The data are given in Table V.

In Experiment 12 (Table VI) a drop in the respiratory rate from 22 to 11 was accompanied by a decrease in tidal air from 70 cc. to 49 cc. and a decrease in minute volume from 1.56 liters to 0.48 liter. The animal breathed room air, and, as could be anticipated from the great reduction in pulmonary ventilation, developed a severe anoxemia The CO_2 content of the serum rose from 29.13 mM to 32.48 mM, the pCO_2 from 51.1 mm. Hg to 70.0 mm., and the pH dropped from 7.36 to 7.26. The period of resistance was 96 minutes. When the resistance was removed the respiratory rate returned to within five

TABLE V.

Experiment 11. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood O_2 content	Arterial blood O_2 capacity	Arterial blood Saturation
				cc	*liters*	*mM*	*mm Hg*		*mM*	*mM*	*per cent*
Control period	12 08 to 12 39	Room air	25	85	2 12	26 62	45 15	7 37	6 35	6 68	95 1
During resistance	12 39 to 2 30	Room air	12	122	1 46	25 87	40 23	7 41	6 69	7 15	93 6
After release	2 30 to 3 12	Room air	24	110	2 64	24 51	35 72	7 44	6 24	6 59	94 7

Weight of animal, 10 5 kilos.
Total barbital-sodium, 0 32 gm per kilo body weight.
Cross-section area of tap finally reduced to 0 1 sq. cm
Positive intratracheal pressure, 13 cm H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1 51.

breaths of the control level. Coincident with the recovery in rate there was an increase in tidal air and minute volume, with the result that the anoxemia was completely relieved and the pCO_2 and pH returned to their previous values.

In Experiments 1B and 10 (Table VII) there was likewise a noticeable decrease in respiratory rate and minute volume.

TABLE VI.

Experiment 12. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc	*liters*	*mM*	*mm Hg*		*mM*	*mM*	*per cent*
Control period	12 40 to 1 14	Room air	22	70	1 56	29 13	51 1	7 36	7 01	7 53	93 1
During resistance	1 14 to 2 50	Room air	11	49	0 48	32 48	70 00	7 26	4 41	8 18	53 9
After release	2 50 to 3 31	Room air	17	84	1 42	28 71	48 23	7 38	7 43	7 70	96 5

Weight of animal, 11.5 kilos.
Total barbital-sodium, 0 30 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Positive intratracheal pressure, 12 cm. H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1 14.

The postmortem examinations in this group of experiments brought out the following points: None of the animals showed pleural effusions, frothy fluid in the trachea, edema of the mediastinal tissues, or gross edema of the lungs. The color of the lung surface in all instances was a diffuse coral-pink, with the same slight variations in intensity as were noted in the previous series. Perhaps the most striking observation was a complete absence of hypostatic congestion in Ex-

TABLE VII.

Experiments 1B and 10. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Experiment 1B					Experiment 10				
	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min	Tidal air	Minute volume
				cc	liters				cc.	liters
Control period	3 15 to 3 43	90–95 per cent O_2	12	166	2 03	1 40 to 2 22	Room air	16	162	2 53
During resistance	3 43 to 6 07	90-95 per cent O_2	9	184	1 59	2 22 to 4 10	Room air	9	138	1.24
After release	6 07 to 6 26	90–95 per cent O_2	8	200	1 68		Room air	—*	—	—

Weight of animal, 13.5 kilos.

Total barbital-sodium, 0 32 gm per kilo body weight.

Cross-section area of tap finally reduced to 0.1 sq cm

Positive intratracheal pressure not recorded.

Duration of greatest resistance, 20 min.

Lung-heart ratio, not determined.

Weight of animal, 11 kilos.

Total barbital-sodium, 0.37 gm. per kilo body weight

Cross-section area of tap finally reduced to 0.1 sq. cm.

Positive intratracheal pressure, 10 cm. H_2O

Duration of greatest resistance, 74 min.

Lung-heart ratio, 1.04

* The resistance was not released in this experiment

periments 1B and 12, with only a slight degree of hypostasis in Experiments 10 and 11. The lung-heart ratios, calculated in three out of the four instances (Experiments 10, 11, and 12) were 1.04, 1.51, and 1.14, respectively. All of these figures are lower than those

obtained with resistance in the inspiratory phase, though at times the difference is very slight.

These experiments may be summarized thus: Resistance to expiration slows the respiratory rate and limits the minute volume of pulmonary ventilation. These changes may or may not be accompanied by a retention of carbon dioxide and a low oxygen saturation of the arterial blood. The effects come on suddenly when the cross-section area of the tap is reduced to 0.1 sq. cm. With removal of resistance all functions return to their normal, or approximately normal, levels. No characteristic pulmonary pathology occurs as the result of resistance to expiration.

DISCUSSION.

The second group of experiments is easier of interpretation than the first. With resistance to expiration there is a mechanical limitation to pulmonary ventilation which may result in carbon dioxide retention and insufficient oxygenation of the blood in the lungs. When the mechanical limitation is removed both the breathing and the state of the blood return to normal and no apparent damage to the lungs has occurred.

The interpretation of the first group of experiments, those dealing with resistance to inspiration, is not so clear. Here, too, we have a mechanical limitation to pulmonary ventilation, but the result in this case is rapid and shallow breathing rather than slow and shallow. There is, as in the other group, inadequate ventilation of the blood. But, even after the resistance had been removed, in many experiments the respiratory rate continued to be rapid, and in some this was true even though the blood had returned to the state found during the control period.

What has happened to the animal which causes it to maintain a rapid respiratory rate in spite of the fact that no longer is there any resistance to the free passage of air into the lungs? The explanation must be sought for in alterations to one of the organ systems having to do with respiratory rhythm. Has the respiratory center itself been damaged or fatigued? There does not appear to be any direct evidence for this assumption. The more or less prolonged period of anoxemia and acidosis which existed in the inspiratory ex-

periments was present in one of the expiratory experiments as well, and yet in this animal there was no accelerated rate after release of resistance. Have the muscles which have to do with inspiration become fatigued as the result of resistance? This hardly seems to be a plausible explanation because in none of the experiments was there a fall in the negative pressure produced in the trachea. Such a fall would have suggested a lessened effort at expansion of the lungs.

The explanation may be sought for more probably in the state of the lungs themselves. The decreased expansion may give rise to a state of pulmonary congestion and this, together with the heightened negative pressure, may result in a seepage of fluid into the pulmonary parenchyma and pleural sacs, as has been suggested by Graham (10). Indeed, it has been experimentally shown by Huggett (11) that inspiratory obstruction increases the minute and stroke volumes of the heart, while expiratory obstruction produces a reverse effect. An augmented blood flow through the lungs may be responsible for the congestion and fluid transudation which were actually observed. It was not unlike that seen in dogs with multiple experimental emboli of the pulmonary capillaries and arterioles (2), nor unlike the changes found after clamping and releasing the artery to one lung (4). In these conditions, too, persistent rapid and shallow breathing occurred.

The local changes in the lung may then perhaps be regarded as responsible for this phenomenon. The normal Hering-Breuer stimuli are increased and predominate. Whether this in itself is evidence of fatigue of the respiratory center, as Haldane believes, is a matter for conjecture.

SUMMARY AND CONCLUSIONS.

1. A study has been made of the effects of resistance to respiration in the inspiratory and expiratory phases.

2. Resistance to inspiration caused an increase in respiratory rate, a decrease in tidal air, and in most instances a severe limitation of the minute volume of pulmonary ventilation. Anoxemia and acidosis accompanied these changes.

3. When resistance was removed the respiratory rate continued to be rapid, but the tidal air and minute volume increased. As a result of this there was a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia.

3. Resistance to expiration slowed the respiratory rate and produced a constant decrease in the minute volume of pulmonary ventilation. Anoxemia and carbon dioxide retention occurred, but were less pronounced than in the inspiratory experiments. Release of resistance to expiration resulted in a return of all functions to their normal, or approximately normal, levels.

4. A difference in the gross pulmonary pathology found at autopsy in these two types of experiments has been described, and an attempt has been made to correlate changes in function with changes in structure.

5. No direct evidence has been supplied for the liability to fatigue of the respiratory center.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 961–968.]

VARICELLA IN MONKEYS.

NUCLEAR INCLUSIONS PRODUCED BY VARICELLA VIRUS IN THE TESTICLES OF MONKEYS.

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(Received for publication, September 3, 1926.)

The lesions observed in testicles of monkeys inoculated with emulsified human varicella papules and vesicles have been described in a previous paper (1) In the experiments reported at that time several species of monkeys were employed and also rats, rabbits, guinea pigs, and chickens. Significant lesions, nuclear inclusions, were found only in the testicles of African vervets 5 and 6 days after inoculation, and not in other inoculated tissues of the same animals, nor in the other experimental animals. Furthermore, similar inclusions were not found in the testicles of a vervet inoculated with normal skin. The inclusions were the eosin-staining nuclear bodies which are consistently associated with certain virus diseases and which, regardless of their nature, indicate to many workers the presence of a virus. Therefore, in view of these facts, it was deemed not unlikely that the acidophilic nuclear inclusions in the vervets' testicles were manifestations of the presence of a virus. The nature of the virus had not been studied at the time of the previous report. Recently, however, experiments were performed to obtain, if possible, additional information concerning the suspected virus and it is with the results of this work that the present paper deals.

Methods and Materials.

Monkeys Employed.—It was impossible to obtain vervets (*Cercopithecus lalandi*). A search for susceptible animals was made among other *Cercopithecus* monkeys. A few experiments showed that green monkeys (*Cercopithecus sabæus*) very closely allied to vervets were satisfactory for the work. It is essential that the monkeys be young, and all animals in which spermatogenesis had been established were discarded.

Inoculations.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5–1.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of Locke's solution, non-immune serum, or immune serum. The mixtures were then injected into monkeys. More than 45–60 minutes never elapsed between the collection of the virus from the patients and its inoculation into animals. All monkeys were inoculated intratesticularly (0.2–2.5 cc.). The non-immune serum was obtained from varicella patients usually within the first 72 hours of the disease. The immune serum was obtained from convalescent varicella patients 14–22 days after the onset of the disease.

Removal and Examination of Testicles.—In previous experiments (1) it was determined that nuclear inclusions were present in the testicles on the 5th and 6th days after inoculation. Therefore in the experiments reported at the present time the monkeys were castrated* on the 5th day. Testicles removed for histological studies were fixed in Zenker's fluid with 5 per cent acetic acid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (2), Lipschutz (3), Goodpasture (4), and others.

EXPERIMENTAL.

The experiments to be reported were conducted to determine whether the eosin-staining nuclear inclusions in monkeys' testicles inoculated with emulsified varicella papules and vesicles are specifically associated with the virus of varicella. In connection with this phase of the work six experiments, consisting of neutralization and reinoculation tests, were performed, and a detailed account of each is given below.

Experiment 1.—Monkeys L and M, green. December 23, 1925. 2 lesions were removed from each of 3 varicella patients, Cases 18, 19, and 20, 3 days after the onset of the disease. The papules and vesicles were emulsified together and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Case 18 was bled on the 3rd day of the disease for non-immune serum; Case 17 was bled for immune serum 17 days after onset of the disease. Equal amounts of the sera, not inactivated, were mixed respectively with the two portions of emulsified papules and vesicles. The mixtures were injected immediately

* All operative procedures were conducted under ether anesthesia.

into 2 green monkeys as follows: Monkey L, 0.25 cc. of virus and immune serum in each testicle; Monkey M, 0.25 cc. of virus and non-immune serum in each testicle. The 4 testicles, removed 5 days later, were fixed and examined in the usual way for the presence of eosin-staining nuclear inclusions.

Although nuclear inclusions were not found in the testicles of Monkey L inoculated with virus and immune serum, typical ones were observed in the testicles of Monkey M inoculated with virus and non-immune serum.

Experiment 2.—Monkeys N and O; green. January 4, 1926. 5 lesions, vesicles and papules, removed from a varicella patient, Case 22, 48 hours after the appearance of the rash were emulsified and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Non-immune serum was also obtained from Case 22 and was inactivated at 56°C. for 30 minutes. Convalescent serum from Case 17, collected and inactivated, December 23, 1925, was used as the immune serum. Equal amounts of the sera were mixed respectively with the two portions of virus emulsion. 2 green monkeys were inoculated immediately; Monkey N, 0.25 cc. of virus and immune serum in each testicle; Monkey O, 0 25 cc. of virus and non-immune serum in each testicle. Monkey N also received 3 cc. of the convalescent serum intraperitoneally; Monkey O, 3 cc. of the non-immune serum in a similar manner. 5 days later the monkeys were castrated. The testicles were fixed, sectioned, and examined in the usual way for the presence of nuclear inclusions.

Nuclear inclusions were not observed in the testicles of Monkeys N and O. No explanation of their absence from the testicles of Monkey O has been found.

Experiment 3.—Monkeys P and Q; green. January 8, 1926. 5 lesions, vesicles and papules, were removed from varicella patients,—2 from Case 24 on the 4th day of the disease, 3 from Case 25 within 36 hours after the appearance of the rash,—and ground up together The emulsified material was taken up in 0.5 cc. of Locke's solution and divided into equal portions. The inactivated non-immune serum was a mixture of sera collected from Cases 18, 22, 24, and 25 on the 3rd, 2nd, 4th, and 2nd days of the disease respectively. The inactivated immune serum was a mixture of sera collected from Cases 17, 18, 21, and 23 on the 17th, 16th, 14th, and 14th days respectively after the appearance of the rash. Equal amounts of the non-immune and immune sera were mixed respectively with the two portions of virus emulsion. The mixtures were injected into 2 green monkeys as follows: Monkey P, 0.25 cc. of virus and immune serum in each testicle; Monkey Q, 0.25 cc. of virus and non-immune serum in each testicle. Monkey P also received 7 cc. of the immune serum intraperitoneally; Monkey Q,

7 cc. of the non-immune serum in a similar manner. 5 days later the testicles were removed, fixed, and examined in the usual way for the presence of nuclear inclusions.

Eosin-staining nuclear inclusions were found in the sections from Monkey Q inoculated with virus and non-immune serum. None were seen, however, in the sections from Monkey P inoculated with virus and immune serum.

Experiment 4.—Monkey A (1); vervet. November 12, 1924. 10 cc. of blood collected from a varicella patient, Case 1, 36 hours after the appearance of the eruption, was injected intravenously into Monkey A. Fluid from 30 vesicles was also collected at the same time and injected intradermally in left eyelid, in left and right thighs, and in right side of abdominal wall. While under observation the animal showed no manifestations suggestive of chicken-pox. No tissue was removed at this time for histological study.

Monkey A (1) was inoculated again, April 7, 1925. Emulsified varicella vesicles and papules from Cases 13 and 14 were injected into the right testicle. 6 days later the testicle was removed and fixed. Nuclear inclusions were not found in the sections.

Monkey A; Monkey R, green. January 9, 1926. 3 lesions were removed from varicella patients,—2 from Case 26 on the 2nd day of the disease, 1 from Case 27 on the 4th day of the disease,—emulsified, taken up in 0.5 cc. of Locke's solution. 0 25 cc. of the emulsion was injected into the left testicle of each monkey. Monkey R also received 7 cc. of fresh unclotted blood from Case 26. 5 days later the inoculated testicles were removed and fixed. A search was made in the usual manner for the presence of nuclear inclusions.

Monkey A was considered to be immune because of the two previous inoculations. Monkey R was normal and approximately as old as Monkey A. Nuclear inclusions were found in the normal testicle inoculated with varicella virus. None were seen, however, in the immune testicle inoculated with the same virus.

Experiment 5.—Monkey R, green. January 9, 1926. Monkey R was employed in Experiment 4. At that time the left testicle was inoculated with varicella virus. It was removed 5 days after the inoculation and sections of it showed nuclear inclusions.

Monkeys R, S, and T; green. February 3, 1926. 2 papules and 2 vesicles were removed from a varicella patient, Case 28, within 48 hours after the appearance of the rash, emulsified, and taken up in Locke's solution. There was 0.75 cc of emulsion. The non-immune serum was collected from Case 25 on the 2nd day of the disease and inactivated. The immune serum was collected from

the same patient 12 days later and inactivated. 0.25 cc. of the virus emulsion was inoculated in the right testicle of Monkey R. 0.25 cc. of the emulsion was mixed with 0.25 cc. of the non-immune serum and then half of the mixture was injected into each testicle of Monkey S. 0.25 cc. of the virus emulsion was mixed with an equal amount of immune serum and then half of the mixture was injected into each testicle of Monkey T. In addition to the above injections Monkeys S and T received intraperitoneally 3.5 cc. of the non-immune and immune sera respectively. The testicles were removed and fixed 5 days later. A careful search through numerous sections revealed the presence of nuclear inclusions in both testicles of Monkey S. None were seen, however, in the testicles of Monkeys R and T.

Experiment 5, in which reinoculation and neutralization tests were conducted simultaneously, is especially interesting. Monkey R's left testicle inoculated with varicella virus, January 9, 1926, showed nuclear inclusions 5 days later. In the right testicle, however, inoculated 25 days later with varicella virus no inclusions were found. The non-immune and immune sera used in the neutralization tests were obtained from the same patient; the former on the 2nd day of the disease, the latter on the 14th. The non-immune serum mixed with virus did not prevent the formation of nuclear inclusions in the testicles of Monkey S. On the other hand, the immune serum inhibited the production of inclusion bodies in the testicles of Monkey T.

Experiment 6.—Monkeys U and V; green. April 6, 1926. 5 lesions, vesicles and papules, were removed from a varicella patient, Case 30, within 48 hours after the appearance of the eruption, and emulsified. The volume of the emulsion was made up to 0.5 cc. with Locke's solution. Patient 30 also supplied the non-immune serum. The immune serum was obtained from Case 29 on the 22nd day after the onset of the disease. Both sera were fresh and not inactivated. 0.25 cc. of each serum was mixed with equal amounts of the virus emulsion. The mixtures were injected immediately into 2 green monkeys as follows: Monkey U, 0.25 cc. of virus and immune serum in each testicle; Monkey V, 0 25 cc. of virus and non-immune serum in each testicle. In addition to the above injections, the monkeys received intraperitoneally 3 cc. of the immune and non-immune sera respectively. The monkeys were castrated 5 days later and a search for the presence of nuclear inclusions in the testicles was made in the usual way.

Eosin-staining nuclear inclusions were found in both of Monkey V's testicles inoculated with a mixture of virus and non-immune serum. On the other hand, inclusion bodies were not seen in Monkey U's testicles inoculated with a mixture of virus and immune serum.

The results of the six experiments described above are summarized in Table I.

TABLE I.

Summary of the Results of Reinoculation and Neutralization Tests.

Experiment	Monkey	State of monkey	Inoculum	Nuclear inclusions
1	L	Normal	Virus + convalescent serum	−
	M	Normal	Virus + non-immune serum	+
2	N	Normal	Virus + convalescent serum	−
	O	Normal	Virus + non-immune serum	−
3	P	Normal	Virus + convalescent serum	−
	Q	Normal	Virus + non-immune serum	+
4	A	Actively immunized	Virus	−
	R	Normal	Virus	+
5	R	Actively immunized	Virus	−
	T	Normal	Virus + convalescent serum	−
	S	Normal	Virus + non-immune serum	+
6	U	Normal	Virus + convalescent serum	−
	V	Normal	Virus + non-immune serum	+

+ indicates presence of nuclear inclusions in testicles.
− " absence " " " " "

DISCUSSION.

Neutralization and reinoculation tests are the accepted means employed to identify viruses and have been extensively used in investigations concerning vaccine virus (5), variola virus (5), poliomyelitis virus (6), Virus III (7–9), herpes virus (10, 11), and others. Technical details of the tests may vary somewhat with each virus, yet in every instance the usefulness of the tests is dependent either upon the specific virucidal properties of an immune serum or upon a specific refractory state of an immune animal.

Many viruses produce characteristic macroscopic lesions in animals, or cause marked changes in their condition. Such alterations and lesions serve as indications of virus activity. Under proper con-

ditions the occurrence of these changes is specifically prevented either by mixing the virus with a homologous immune serum prior to the inoculation of the animal or by injecting the virus alone into an animal previously immunized by means of the same virus. In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as indicated by the presence of inclusion bodies in the nuclei and cytoplasm of injured cells. Sometimes, however, the microscopic changes are the only manifestation of the presence of a virus (1, 12). When such a condition arises, there is no obvious reason why the microscopic changes, inclusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed.

Many workers believe that the eosin-staining nuclear inclusions are the manifestations of the presence of certain filterable viruses, including the virus of varicella. Consequently at the time of the previous report (1) there were grounds for the belief that the nuclear inclusions found in the monkeys' testicles inoculated with emulsified varicella papules and vesicles were produced by the action of varicella virus. Proof of this, however, was obtained only recently by means of the experiments reported above, and consists, in brief, of the following considerations. Nuclear inclusions were not found in monkeys' testicles inoculated with a mixture of varicella virus and convalescent varicella serum. On the other hand, they were found in testicles inoculated with a mixture of virus and non-immune serum collected from varicella patients early in the disease. Furthermore, the inoculation of one testicle with varicella virus prevented the formation of nuclear inclusions in the other one when it was inoculated at a later date with the same virus.

So far as is known, the evidence presented in this paper is the only definite proof on record that experimental animals are susceptible in any way to the action of varicella virus.

CONCLUSION.

The eosin-staining nuclear inclusions found in the monkeys' testicles inoculated with emulsified tissue of human chicken-pox lesions are specifically associated with the virus of varicella.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol. xlv, No. 6, pp. 993–1002.]

FURTHER OBSERVATIONS CONCERNING GROWTH REQUIREMENTS OF HEMOPHILIC BACILLI.

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(Received for publication, February 21, 1927.)

Hemophilic bacilli form a heterogeneous group of bacteria. Observations concerning their growth requirements and the hemolysis caused by some strains have been reported. By means of these observations a subdivision of this group of organisms has become possible.

Pfeiffer (1), 1893, first described *B. influenzæ*. He was unable to cultivate the bacillus in the absence of blood pigment. Grassberger (2), 1898, reported that Pfeiffer's bacillus in symbiosis with *Staphylococcus aureus* was able to multiply on media containing hematin Olsen (3), 1920, showed that growth of *B. influenzæ* occurred only on media giving a positive peroxidase reaction. Fildes (4), 1920, obtained satisfactory growth of Pfeiffer's bacillus on media enriched by means of a peptic digest of blood. Later, in 1921, he also showed (5) that in this digest each of two factors was essential for the growth of the organism; one was present in the clear supernatant fluid, the other occurred in the brown hematin deposit Avery and Thjøtta (6, 7), 1921, in a series of papers, reported that two accessory factors are necessary for the cultivation of influenza bacilli. These factors were designated as X and V. The V substance is relatively heat-labile and is found in blood, in cultures of many bacteria, and in yeast and vegetable cells. The X substance is heat-stable, gives a positive peroxidase test, and is found in blood and raw potatoes. Rivers and Poole (8), 1921, showed that two accessory factors are essential for the growth of influenza bacilli; one is present in filter-sterilized yeast extract, the other in autoclaved extract of blood. Rivers (9, 10), 1922, reported that Friedberger's *B. hemoglobinophilus canis* requires only the X accessory factor, and that from patients 2 strains of bacilli had been obtained that require only the V accessory factor. The strains requiring only V factor were designated *B. parainfluenzæ*. Rivers and Bayne-Jones (11), 1923, obtained from throats of cats 6 strains of influenza-like bacilli that required only the V factor.

Pritchett and Stillman (12), 1919, described a Gram-negative, aerobic, non-motile, hemophilic bacillus and designated it as Bacillus "X." Stillman and Bourn (13), 1920, reported further observations concerning the characteristics of Bacillus "X." Rivers (14), 1921, suggested that these organisms be called hemolytic influenza bacilli. Rivers (10), 1922, on testing a hemolytic strain found that

it required both X and V growth accessory factors. Fildes (15), 1924, reported that 13 of 14 strains of hemolytic influenza bacilli required only the V factor. The remaining strain, however, required both X and V. The former strains grew on ordinary media in symbiosis with staphylococci, and also with *B. hemoglobinophilus canis* on media containing the X factor. This fact indicated to Fildes that *B. hemoglobinophilus canis* synthesizes the V factor.

Particular interest in the hemophilic bacilli was again aroused by the occurrence in New York during the spring of 1926 of a respiratory infection resembling mild influenza. Attempts were made to determine (1) how frequently hemophilic bacilli occur in the upper respiratory tract of patients not necessarily suffering from influenza, and (2) what variations in growth requirements exist among strains of these bacilli obtained from such sources. The results of this investigation are dealt with in the present paper.

Methods and Materials.

Autoclaved meat infusion broth was used as a basic medium. Filter-sterilized yeast extract, prepared in the manner described by Avery and Thjotta, supplied the V factor. 0 5 cc of the extract was added to 5 cc. of broth. A solution of hematin, prepared in the manner described previously (11), supplied the X factor. Dilutions, 1 to 10,000, of this solution gave positive reactions for the presence of peroxidase. When the presence of the X factor was desired in the medium, 1 cc of the hematin solution was added to each 100 cc. of broth. The broth or yeast extract alone never gave positive peroxidase tests.

Bacilli used in this work were obtained from the throats of patients by means of cultures on fresh 2 per cent rabbit blood agar plates. Hemolytic and non-hemolytic Gram-negative bacilli isolated from these plates were carried in stock on blood agar slants A strain from the spinal fluid of a patient with influenzal meningitis and 1 from a pneumonic lung were also studied.

The bacilli to be tested were removed from blood agar slants by means of a platinum loop and transferred to tubes of broth containing yeast extract, hematin, and yeast extract and hematin respectively. In the case of non-hemolytic strains that grew only in broth containing both factors (X and V), and of hemolytic strains that required the addition of only the V factor, macroscopic evidence of growth was considered sufficient and no subcultures on solid media were made. Other strains, however, were tested more completely in regard to their growth requirements.

EXPERIMENTAL.

From 46 patients, 34 strains of so called hemophilic bacilli were isolated. In Table I the sources from which they were obtained and

the frequency with which they occurred in certain groups of cases are indicated. In some of the groups too few cases were studied to permit of a comparison between the groups. Omitting the 4 cases of influenza, however, 42 cases of other diseases were examined and the results are interesting and significant. From these 42 cases, 18 strains of non-hemolytic bacilli (43 per cent) and 11 strains of hemolytic ones (26 per cent) were obtained. Although the patients did not have influenza, 29 of them (69 per cent) harbored hemophilic bacilli in their throats. These findings are in accord with those of other workers who have shown that hemophilic bacilli are likely to be encountered very frequently during periods of epidemic respiratory infections.

TABLE I.

Sources of the Hemophilic Bacilli Studied.

Type of case	Number examined	Number of non-hemolytic strains isolated	Number of hemolytic strains isolated
Chicken-pox	27	8	7
Measles	9	6	3
Influenza	4	1	4
Miscellaneous	6	4	1
Total .	46	19	15

The 34 strains of bacilli were tested in the manner described above to determine their need of growth accessory factors, V and X. The results are summarized in Table II.

From Table II it is obvious that 17 of the non-hemolytic strains required both growth accessory factors (X and V). They were similar in every respect to organisms usually accepted as influenza bacilli (Pfeiffer). There were 2 non-hemolytic strains, however, that grew well when yeast extract (V) alone was added to the broth. These organisms were carried through 7 successive transplants in this medium Subcultures were pure. In broth alone or in broth plus hematin, with 0.25 cc. amounts for transfers, no viable bacilli were found by means of subcultures on blood agar after the 2nd transfer. Neither strain produced indole. Both reduced nitrates to nitrites.

Each grew diffusely in liquid media. These bacilli corresponded to Rivers' *B. parainfluenzæ*.

There were 10 hemolytic strains that grew well in broth to which only yeast extract (V) was added. These bacilli were fairly uniform in type. Cultures of them in liquid media usually exhibited flocculi similar to the ones seen in streptococcus cultures. On blood agar plates, colonies of the bacilli were surrounded by large zones of hemolysed red blood cells, were slightly opaque, and were often firm enough to be pushed about intact on the surface of the medium.

TABLE II.

Summary of Results Obtained Concerning the Growth Requirements of the Hemophilic Bacilli.

Type and number of strains	Broth + yeast extract	Broth + hematin	Broth + yeast extract + hematin	2 per cent rabbit blood agar
17 non-hemolytic	−	−	+	+
2 non-hemolytic	+	−	+	+
10 hemolytic	+	−	+	+
3 hemolytic	−	−	+	+
2 hemolytic	−	−	−	+
1 *B influenzæ*, control	−	−	+	+
1 *B. parainfluenzæ*, control (original Rivers strain)	+	−	+	+
1 *B. hemoglobinophilus canis*, control	−	+	+	+

The + sign indicates visible growth. The − sign indicates no growth. Yeast extract supplied the V factor; hematin, the X factor.

Upon microscopic examination the bacilli were found to be exceedingly pleomorphic and usually larger than Pfeiffer's bacillus. In order to maintain stock cultures on blood agar slants it was necessary to make transfers more frequently than at weekly intervals.

Three hemolytic strains required both X and V growth accessory factors. They were carried through 7 successive transplants in broth containing yeast extract and hematin. In the later transfers, successful transplants were obtained by means of a platinum loop. In broth containing yeast extract or hematin alone no viable bacilli were present after the 3rd or 4th transfer. 2 other hemolytic strains that grew

well in rabbit blood broth were not viable after 3 or 4 transfers in broth containing both yeast extract and hematin. It is possible, however, that after a longer period of cultivation on artificial media, results with these 2 strains might have been similar to those obtained with the 3 strains described above. On blood agar plates colonies of these 5 strains were surrounded by very narrow zones of hemolysed red blood cells. They were small, transparent, moist, and soft. In fluid media the bacilli always grew diffusely. All 5 strains reduced nitrates to nitrites. One produced indole. Upon microscopic examination these organisms resembled Pfeiffer's bacillus. Weekly transplants of the organisms on blood agar were found to be sufficient for maintenance of stock cultures.

From the results of the experiments already described, it is evident that some hemophilic bacilli hemolyse red blood cells, while others do not, that some require both X and V growth accessory factors, while others require either X alone or only V. Fildes showed that organisms requiring only V, or both X and V, multiplied in symbiosis with *B. hemoglobinophilus canis* on media containing only X. From this observation he concluded that the latter organism synthesized V and supplied it to the bacilli needing it for growth. Since some hemophilic bacilli require the addition of only V to the media used for their cultivation, and since it has been shown that many bacteria capable of growing on ordinary media, *e.g.* staphylococci, give a positive peroxidase reaction, experiments were performed to determine (1) if the hemophilic bacilli capable of growing on media to which only V is added give a positive peroxidase test, and (2) if they are able to provide the X factor to bacteria needing it for growth. The results of these experiments will be described below.

In the experiments to determine whether the bacilli requiring the addition of only V to the media give a positive test for peroxidase, the following strains were employed: Rivers' original *B. parainfluenzæ*, 2 non-hemolytic strains which were described above and which will be spoken of as *B. parainfluenzæ* Nos. 2 and 3, and 1 hemolytic strain. The 4 strains of bacilli were grown in broth to which yeast extract had been added. Cultures 24 hours old were used in every instance. A portion of each culture was saved for tests. The remaining portion was centrifuged and the sedimented bacilli were resuspended in salt solution This procedure was repeated twice. A portion of the original culture, the last supernatant salt solution, and the washed bacilli suspended in salt solution were

tested for the presence of peroxidase in the usual way by means of benzidine. The suspension of bacilli was much denser in the salt solution than in the original broth cultures. The results of the experiments are shown in Table III.

The results of the experiments summarized in Table III indicate that the hemophilic bacilli requiring the addition of only yeast extract (V) to the media give a positive test for peroxidase (X) in spite of the fact that the medium in which they are grown gives a negative one.

B. hemoglobinophilus canis requires the addition of X to its media, yet apparently produces V. *B. parainfluenzæ* and some of the hemolytic bacilli require the addition of V to their media, but give a positive test for peroxidase (X). In view of these facts it seemed of interest to ascertain whether the organisms requiring the addition of X alone and the ones needing the addition of only V are capable of supplementing the growth accessory factors for each other when seeded together in media to which neither X nor V has been added. The manner in which this question was investigated is described below.

TABLE III.

Results of Benzidine Tests for Presence of Peroxidase.

Strains tested	Original culture	Supernatant salt solution	Washed bacilli
B. parainfluenzæ, No. 1	±	–	+
B. parainfluenzæ, No. 2	–	–	±
B. parainfluenzæ, No. 3	–	–	±
Hemolytic bacillus	–	–	±

The ± sign indicates a faintly positive reaction. The + sign indicates a positive reaction. The – sign indicates no reaction.

Autoclaved meat infusion broth and meat infusion agar to which no growth accessory factors had been added were the media employed. *B. hemoglobinophilus canis* requiring the addition of X alone, *B. parainfluenzæ*, Nos. 1, 2, and 3, needing the addition of only V, and a hemolytic bacillus also requiring the addition of only V were the organisms studied. When a liquid medium was used transfers were effected by means of pipettes, 0.25 cc. being the size of the inoculum. Cultures were incubated 72 hours between transplants. Subcultures on blood agar plates were employed to determine the viability and the types of the bacilli in the different tubes. The ability of the bacilli to grow alone or together under the conditions mentioned is indicated in Tables IV and V.

TABLE IV.

Summary of Results of Fxperiments Concerning Symbiosis of Hemophilic Bacilli in Meat Infusion Broth.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	+	?	–				
2. *B. parainfluenzæ*, No. 1	X	+	?	?	–			
3. *B. parainfluenzæ*, No. 2	X	+	–					
4. *B. parainfluenzæ*, No. 3	X	+	?	–				
5 Hemolytic bacillus	X	+	?	–				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The – sign indicates no growth when subcultures were made on blood agar. The + sign indicates visible growth of bacilli of the type or types with which the series was started

TABLE V.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli on meat Infusion Agar.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	?	–					
2. *B. parainfluenzæ*, No. 1	X	?	–					
3. *B. parainfluenzæ*, No. 2	X	?	–					
4. *B. parainfluenzæ*, No. 3	X	?	–					
5. Hemolytic bacillus	X	?	?	–				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The – sign indicates no growth when subcultures were made on blood agar. The + sign indicates a visible growth of bacilli of the type or types with which the series was started.

From the results shown in Tables IV and V, it is obvious that bacilli requiring the addition of X alone and those requiring the addition of only V to their media grow well in symbiosis on media to which neither X nor V has been added. This fact indicates that these organisms are capable of supplementing the growth accessory factors for each other when grown together under the conditions of the experiments.

DISCUSSION.

The results of experiments reported in the first part of the paper support the findings previously recorded by Rivers and Fildes concerning the growth requirements of non-hemolytic and hemolytic hemophilic bacilli. Furthermore, these results indicate that a subdivision of this group of bacilli based upon their growth requirements is not only possible but also practicable. The majority of the non-hemolytic strains require the addition of both X and V to their media, while a few need the addition of only V. On the other hand, most hemolytic strains require the addition of only V to their media, while some need the addition of both V and X. It is true that 2 hemolytic strains were encountered that were susceptible to cultivation in blood broth but not in broth containing yeast extract and hematin. In view, however, of their recent isolation and of the difficulty experienced with 3 similar strains that required both factors, it seems justifiable to consider the 5 strains as members of one group, which, in its general characteristics, is more closely related to Pfeiffer's bacillus than to the other hemolytic bacilli under discussion.

The results of the investigation reported in the latter part of the paper apparently indicate that strains of hemophilic bacilli requiring the addition of only V or X to their media are capable of supplying, or acting as, X or V respectively to an extent sufficient for the needs of other bacilli that require the addition of these factors to their media. This fact supports the view that organisms which do not require the addition of either growth accessory factor to their media only multiply on such media because they are capable of synthesizing these factors.

Discussions have arisen in regard to the number of bacilli that should be included in the hemophilic group. Some workers contend

that this group of organisms should not be regarded as hemophilic, inasmuch as all of its members under certain conditions are capable of growth in the absence of blood. There is evidence, also, that in the past similar organisms have been classified under different headings, *e.g.*. Koch-Weeks' bacillus and Pfeiffer's bacillus are identical. Because of the variety of organisms that have been placed in this group any kind of a classification will have some objectionable features. Knowledge, however, recently acquired concerning the growth requirements of the hemophilic bacilli and the ability of some of them to hemolyse red blood cells enables one to outline a relatively simple classification of this heterogeneous group of organisms. Such a classification is presented below. The growth requirements, X and V, of a few bacilli placed in the hemophilic group have not been determined. When this has been accomplished these organisms can then be given their proper place in the classification presented.

Classification of Hemophilic Bacilli.

A. Requiring the addition of V and X to the media.
    B. influenzæ (*Hemophilus influenzæ*).
    1. Non-hemolytic.
    2. Hemolytic.
B. Requiring the addition of only V to the media.
    B. parainfluenzæ (*Hemophilus parainfluenzæ*).
    1. Non-hemolytic.
    2. Hemolytic.
C. Requiring the addition of only X to the media.
    B. hemoglobinophilus canis (*Hemophilus canis*).
D. Requiring the addition of neither X nor V to the media.
    1. *B. pertussis* (*Hemophilus pertussis*).

SUMMARY.

1. 19 strains of non-hemolytic hemophilic bacilli were studied. 17 required the addition of V and X growth accessory factors to their media, 2 required the addition of only V.

2. Of 15 strains of hemolytic hemophilic bacilli examined, 10 were found to require the addition of only V to their media, 3 the addition of V and X in the form of yeast extract and hematin, and 2 the addition of accessory growth factors in the form of blood

3. In media to which neither V nor X had been added true symbiosis was found to occur on growing *B. hemoglobinophilus canis*, requiring the addition of X, with *B. parainfluenzæ*, requiring the addition of V, or with hemolytic strains of bacilli, requiring the addition of only V.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1927, Vol. xlvi, No. 1, pp. 123–131]

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

I. THE PATHOGENIC ACTION OF CULTURE FILTRATES.

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(Received for publication, March 18, 1927)

The strains used in these studies were all isolated from the ileum of young calves passing liquid feces and showing signs of a choleriform disease (scours). Most of the strains were obtained from sick calves, some moribund, immediately after they had been killed, a few after natural death. Several strains from normal calves were introduced for comparative study. They differ from one another in motility, fermentative capacities, the possession of capsules, tendency to mutate, and the like. More detailed descriptions will be given in papers to follow.

B. coli, representing a group, may be regarded as a special type of parasite, restricted to the digestive tube with occasional sallies into those organs or cavities where the protective forces of the host cannot be fully exerted, as in the urinary tract It was thought that any new light which can be shed on its behavior might be useful in interpreting the behavior of the more highly specialized and parasitic paratyphoid group.

In various articles one of us has formulated the hypothesis that in the gradual evolution of pathogenic or invasive types of bacteria, the beginnings of parasitism may have been made possible by a soluble, diffusible toxin, but that in later stages this primary offensive, more or less accidental, mechanism is either partly or wholly suppressed and some different mechanism developed with which the bacteria protect themselves against the body-foreign forces of the host. The process may be regarded as shifting from the destructive, predatory to the parasitic, from the offensive to the defensive type. According to

this hypothesis, *B. coli* represents the early predatory, toxic stage, with, however, a certain specialization towards protection from anti-foreign activities in the digestive tract. It resembles in many respects the cholera vibrio in its activities. In view of the hypothesis presented, a study of any early appearing toxin in cultures seemed the first problem to be attacked.

EXPERIMENTAL.

Effect of a Living Culture on Calves.—In the following pages, the effect of culture filtrates on calves is the chief topic. As a preliminary, the notes on the effect of a living bouillon culture are here introduced.

A calf (No. 307), 32 days old, weighing 104 lbs , received into a jugular vein 2 cc. of a 24 hour bouillon culture of *B. coli* 223. This strain produced a large amount of viscid material, even within 24 hours In 2 days, the entire bouillon became viscid, so that short, cobweb-like threads could be raised from the fluid The calf was seen 25 minutes later. When seen again, 65 minutes after the injection, it was lying on its side, dead, and a mass of white froth about 12 inches long extended from its nostrils on the floor of the stall The autopsy showed the following conditions

Digestive tract pale throughout. Upper respiratory tract, including pharynx and larynx, cyanotic. Tracheal mucosa injected and covered with froth. Lungs large and heavy. Both large caudal lobes are intensely and uniformly congested, the condition bordering on hemorrhage. The condition as to blood content varies from lobule to lobule. The smaller lobes (ventral, cephalic) are far less congested and edematous Subendocardial tissue, left side, around papillary muscles, infiltrated with blood in form of large patches Liver with borders rounded Over large areas there are confluent patches of a dark red color. These, on section, correspond to diffuse hemorrhage into parenchyma similar to the pulmonary lesions. Both kidneys have large dark red areas in the cortex This hemorrhagic condition dips down in a linear way to medulla Besides these radiating lines of hemorrhage, hemorrhagic patches are present, as in liver and lungs Urine from bladder clear, amber-colored, and free from any visible blood tint. It contains a small amount of coagulable protein. In sections of lung, kidney, and liver the described lesions are shown to be due to an intense congestion or filling up of the capillary system associated with and merging into hemorrhage. When 16 days old this calf was fed with a heavy suspension of the same culture without showing disturbance of any kind.

Effect of Filtrates.—In a study of filtrates it was deemed best to restrict the experiments on calves to relatively young cultures. Veal broth containing 1 per cent peptone and 0.1 per cent dextrose was

sterilized in flasks in layers 2 to 3 cm. deep. 48 hours after inoculation the cultures were filtered through Berkefeld filters and the filtrate stored in full bottles at 38–40°F. until used. Any deterioration within several months was not observed

The effect on calves 1 to 2 months old, on calves 6 to 7 months old, and on cows was qualitatively the same when the filtrate was injected into a jugular vein. Subcutaneous injection was without visible effect. After the intravenous injection of 2 cc. of filtrate the first signs in calves were manifest in from 5 to 20 minutes by a slight cough. After this there was a speedy increase in the number of respirations and pulse beats, the former rising to 100, rarely 120 or higher, the latter to 80 or even 100 and above. After 1 or more hours, both declined and the respirations became somewhat jerky. The expirations were usually interrupted and accentuated by a grunt. The temperature rose 1° or 2°C. after the injection but was normal the next day. The respiratory difficulties may in some animals become very great. The mouth is then held open, and saliva dripping from it, the head and neck held horizontal Usually the calf is very restless, lies down and gets up repeatedly, or when very weak it lies on its side with the legs extended

The reaction following the intravenous injection is, as might be supposed, not the same in all calves. A few failed to manifest the acute respiratory distress and the reaction showed itself in muscular tremors and chills In most calves there were repeated discharges of semiliquid feces in addition to the respiratory symptoms The pronounced symptoms usually last 4 to 6 hours Rarely the depression continues over 1 or 2 days The following protocol is inserted to illustrate the time intervals of the several stages of the toxic effects.

10.20 a m. Calf, 34 days old, Holstein female, receives, intravenously, 2 cc. filtrate of a 48 hour culture of *B. coli* 1085.

10.25 a.m. Calf very sick, lies down; respirations 130 Coughs frequently.

10.50 a.m. Respirations 88; pulse 60 Respiratory conditions the same.

11 a.m. Respirations 40. Temperature 38.9°C. Pulse 70

12.15 p.m. Lying with head extended and legs straightened at right angles to body. An expiratory grunt as of some obstruction to expulsion of air.

3.15 p.m. Still lying in the same position. Temperature 39.1°C. A grunt with each expiration but not with any check in the movement Respirations 70

4 p.m. Temperature 39 4°C. Still lying down exhausted. Respiration as before. Copious discharge of feces.

5 p m. Calf standing up. Brighter. Takes its evening food. Temperature 38 9°C

Calf slightly depressed on the following day. Temperature about normal.

In calves 6 to 7 months old the same dose of 2 cc. produced severe reactions. One cow treated with the filtrates intravenously reacted severely after each injection of a dose increasing gradually to 15 cc. The filtrates of five strains of *B. coli* from calf scours distinguishable culturally from one another all produced the same succession of symptoms

The mode of introduction of the filtrate naturally brings the respiratory tissues first under the influence of the toxin. The symptoms indicate an injury of the alveolar epithelium and vascular endothelium leading to increased permeability and transudation of fluid into the alveoli. That the toxin is a capillary poison is furthermore indicated by the lesions found in the fatal case following the injection of a living culture described above, and the following cases in which the autopsy showed the end effect on the lung tissue.

Holstein heifer calf, 48 days old, received into a jugular vein, a mixture of 2 cc. *B. coli* filtrate (1192b) and 6 cc. serum from a cow which had been treated with filtrate. This calf had received intravenously 2 cc filtrate and 2 cc. serum when 38 days old It went through the typical reaction associated with rapid respiration, open mouth and dribbling of saliva, 35 minutes after the injection Dyspnea became pronounced and associated with a grunt during each expiration. The labored breathing continued from 11 a.m until well into the night The calf appeared free from any respiratory difficulties next morning. It was killed 2 days later The viscera were normal with exception of the lungs which displayed an irregularly distributed congestion and small hemorrhagic areas Sections showed the presence of deformed, cup-shaped red cells in small numbers in the alveoli of various regions. Distinct hemorrhagic areas were also present. In some lobules the alveoli contained granular and fibrillated material evidently fibrin. Polymorphs were loosely distributed in small numbers in the alveoli partly enmeshed in the alveolar coagulum. Mitoses of alveolar epithelium were not infrequent. This animal thus was still under the influence of a pulmonary congestion associated with small hemorrhages. There was no evidence of a persisting early or fetal pneumonia

A Guernsey male calf, 4 weeks old, received into a jugular vein 2 cc. of a filtrate of *B coli* 223, the same strain which had been fatal to Calf 307, 7 years ago. The filtrate was prepared from a bouillon culture 48 hours old.

The symptoms referable to the respiratory tract followed in the order described but with unusual intensity. The respiratory dyspnea came on within 30 minutes.

The calf breathed with mouth open. The respirations rose to 112, but dropped to 48 after 4 hours. At this time the calf was too weak to stand The respiratory difficulty increased and the calf died about 5½ hours after the injection. The autopsy immediately after death showed besides a general congestion of the viscera, an intense congestion of the lungs. The right cephalic lobe was completely airless, heavy. On section the parenchyma was evidently filled with blood The left cephalic lobe contained a little air. The interlobular tissue was broadened by hemorrhagic infiltration. The azygos lobe was in part airless through hemorrhages. Both large caudal lobes showed all degrees of congestion, edema, and hemorrhage from the still air-containing caudal tips to the cephalic margins of these lobes.

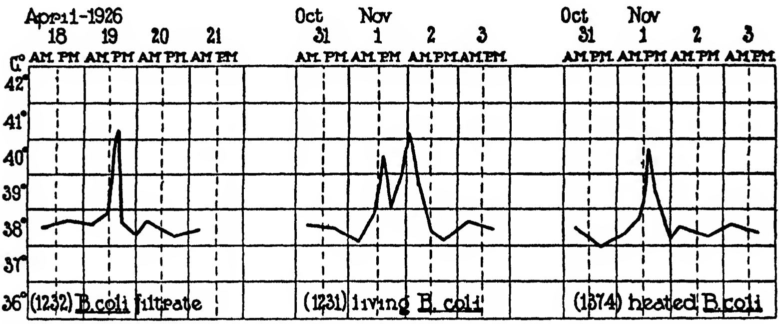
The spleen was enlarged through congestion. The kidneys were moderately congested, with a more deeply injected zone between cortex and medulla Urine dribbling from the urethra during the early hours after injection of the filtrate was clear, slightly alkaline, specific gravity 1 002, with no protein coagulable by heat Urine taken from the bladder soon after death had a specific gravity of 1.015, and contained coagulable protein equivalent to a deposit of 1 3 cc in 10 cc fluid.

Another calf received subcutaneously, at birth, 40 cc serum from a cow treated with *B. coli* filtrates When 29 days old and weighing about 112 pounds, it received into a jugular vein 2 cc filtrate of a 48 hour bouillon culture of *B. coli* 1192a diluted with 3 cc bouillon. The filtrate was 49 days old and it had been stored continuously at 36–40°F in full bottles Symptoms began within 20 minutes and ran the usual course with panting respirations rising to 120 per minute The calf stood with head low, mouth open, and tongue protruding 1½ hours after injection the respirations had fallen to 72 The animal was then very sick and unable to stand Grunts with every expiration. 3½ hours after injection the animal began to be easier Respirations 60, with occasional grunts In 6 hours the reaction was nearly over and 1 hour later the evening meal of milk was taken readily

The calf was killed about 23 hours after injection when in outwardly normal condition. The vessels of neck were severed after stunning the animal The only organs visibly changed were the lungs. These were extensively involved. Three forms of lesions could be observed (*a*) A dark red hepatization due to exudation into alveoli of blood and coagulation there About 8 cm of the free tip of the left cephalic lobe was in this condition. (*b*) Localized congestions and hemorrhages involving one or several lobules. These were scattered through the main (caudal) lobes chiefly (*c*) Petechial hemorrhages 1 to 3 cm apart, chiefly in the small cephalic lobes. The liver showed a patchy congestion visible both on surface and on section. Each liver cell contained one or more fat globules 2 to 5μ in diameter Kidneys slightly congested and distinctly more moist on section than normal. Hyperemia of boundary zone between cortex and medulla Spleen congested but normal markings still visible. Slight patchy hyperemia of upper small intestine Urine about 30 minutes after injection contained about

1.3 per cent deposit of coagulable protein after heating. At autopsy there was only about 0.3 per cent. Urine 12 days before injection was normal.

Sections of fixed and hardened tissue presented nothing noteworthy beyond what is stated in the autopsy notes concerning the liver, spleen, and kidneys. In sections of the lungs, that portion found consolidated at autopsy presented various lesions. Focal hemorrhages were numerous and filled a group of contiguous alveoli with red cells. Forming a background for these hemorrhagic areas, the remainder of the alveoli contained a delicate network of fibrin fibrils holding enmeshed polymorphs in small numbers. The latter cells were brought together in denser groups within alveoli here and there. The other lobes of the lungs showed areas with partly collapsed alveoli and broadened walls as well as occasional small hemorrhages and alveolar fibrin. Polymorphs were thinly distributed throughout, both in the capillary bed and the alveolar lumina.



TEXT-FIG. 1. Temperature reactions in calves following the intravenous injection of *B. coli* and filtrates.

Thus far only filtrates of 48 hour bouillon cultures had been used. The presence of abundant toxin in a 24 hour culture was demonstrated on a calf 26 days old. After the intravenous injection of 2 cc. of the filtrate the sequence of symptoms already described appeared and with an intensity fully equal to that following the 48 hour culture filtrate. The calf was killed within 48 hours of the injection. The lungs still presented signs of the reaction in the form of subpleural hemorrhagic points and scattering congested lobules.

The effect of the intravenous injections on the rectal temperature of calves is shown in Text-fig. 1. The effect of a filtrate of a 48 hour bouillon culture is shown in (*a*). A similar unimodal curve (*c*) is pro-

duced by cultures heated at 60°C. for 30 minutes and therefore sterilized. In (*b*) the bimodal curve following the injection of living cultures may be due to immediate effects of the toxin followed by a temporary multiplication of the injected bacteria and hence a second dose of toxins.

The experiments reported were made with 24 and 48 hour culture filtrates. One test was made with a filtrate of a culture of 1192a incubated 8 days. The layer of bouillon in the flask was about 1 cm. deep. At the end of the incubation, the fluid was quite viscid and hence diluted with an equal volume of normal saline to facilitate passage through a Berkefeld filter.

4 cc. of the filtrate, containing 2 cc. of the original culture fluid, was injected into a jugular vein of a calf 34 days old. The symptoms followed one another as in the preceding cases but with much greater intensity and rapidity. The calf died in 3½ hours after the injection. There was complete hemorrhagic filling up of the entire left lung excepting a narrow margin of ventral and cephalic lobe. On section, the tissue was uniformly dark red, with reddish frothy fluid trickling off. The tissue was heavy but still resilient. The right lung, along median and dorsal region, was in the same condition; the lateral two-thirds of this lung was still partly air-containing with dark red areas in each lobule. Much foamy reddish fluid flowed from cut section of the pinkish regions. The trachea was filled with a reddish froth. There was a moderate congestion of the kidneys and some fat in the liver cells. The mucosa of intestines was only feebly reddened. The spleen was congested and weighed 460 gm.

This preliminary test clearly indicated a rise in the toxicity of the culture fluid due to longer incubation. Whether there is but one toxin involved or others superadded during the longer incubation remains unanswered for the present.

The effect of filtrates on guinea pigs introduced into the peritoneal cavity is relatively slight when compared with the serious effect on calves weighing about 100 pounds. The guinea pig weighing 350 to 600 gm. receiving the calf dose of 2 cc. into the peritoneal cavity reacts only with loss in weight as follows: Within 2 days there is a loss of 35 to 50 gm. in weight. Then there is a recovery so that in 7 days the original weight has not only been regained but added to by 10 to 15 gm. A small portion of this loss may be produced with bouillon alone The injection of smaller doses, up to 0.5 cc., directly into the

heart failed to produce any acute symptoms or later death. In relation to body weight 0.5 cc. into the circulation of guinea pigs is over 30 times, the intraperitoneal dose of 2 cc. over 120 times the calf dose. In an early, fairly comprehensive study of calf scours, E. Joest[1] finding that filtrates of 24 hour bouillon cultures of *B. coli* were non-toxic for guinea pigs after intraperitoneal injection up to 3 cc., concludes that soluble poisonous products are not secreted.

It has already been stated that the subcutaneous injection of filtrates is without appreciable effect. The same is true when living cultures are introduced with the food. Two calves, about 2 months old, were fed *B. coli* in milk without showing any digestive or other disturbances. 500 cc. milk had been heavily seeded with a bouillon culture, warmed, and incubated for 7 hours. Plate cultures indicated ½ billion bacteria per cc. To disguise the flavor, fresh milk was added. The incubated milk coagulated when heated and was strongly acid to litmus.

Owing to obvious difficulties in multiplying experiments on calves only a few have been made bearing other phases. When bouillon cultures were shaken with kaolin and filtered there was no reduction of toxicity. When the filtrate was exposed to 80°C. for 30 minutes the toxicity though decidedly reduced was not completely destroyed.

One calf was treated with a 48 hour culture filtrate of a paratyphoid bacillus from guinea pigs[2] 4 cc in place of the usual 2 cc. were injected into a jugular vein The symptoms following were similar to those produced by the *B. coli* filtrate, but less severe The calf began to cough in 13 minutes In 15 minutes the respirations were 104 In 30 minutes the calf was lying down, with respirations at 80 and evidently labored After 1½ hours, respirations were 60 and dyspnea pronounced Viscid saliva was hanging from the mouth After 3 hours, the calf was lying quiet and apparently without distress It took its milk after 6 hours Next day it was still subdued During the attack the temperature rose 1–1.5°C.

The soluble toxins of the large group of typhoid, paratyphoid, and colon bacilli have interested many observers since these groups were definitely recognized. Much of the work has been done in the paratyphoid group owing to its close relation to outbreaks of meat and

[1] Joest, E., *Z. Tiermed*, 1903, vii, 377.

[2] Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353; and Smith, T., and Nelson, J. B, *J. Exp. Med*, 1927, xlv, 365.

other food poisoning. A fairly complete bibliography has been published recently by Miss Branham.[3] Among the recent papers which bear directly on the subject of this communication is one by Steinberg and Ecker[4] who prepared an antiserum in rabbits towards the soluble toxin with a culture fluid centrifuged but not filtered. The antiserum was tested upon rabbits inoculated with living cultures. The results apparently demonstrated the neutralizing power of the antiserum on the soluble toxin. An analysis of the experiments does not bear out the inference drawn. The rabbits received some living bacteria as antigen and the effect of the antiserum in the test rabbits may have been a suppression of the bacteria injected rather than a neutralization of the soluble toxin.

SUMMARY AND CONCLUSIONS.

The relatively young bouillon filtrates, 24 and 48 hours old, of certain strains of *B. coli* obtained directly from the ileum of scouring calves, were highly toxic for calves about 1 month old, as well as for older calves and cows when given into a vein The symptoms, of panting followed by dyspneic and jerky respiration, indicate some at first obstructive action upon the alveolar and endothelial cells, followed by a greater permeability and eventual filling up of the air spaces with a serous, fibrinous, and hemorrhagic exudate. Similar effects are produced in other organs, such as liver and kidneys, if the toxin reaches them or is formed there by multiplying bacteria. There are no immediate or remote effects resembling those on calves following the intraperitoneal or the intracardiac injection of *B. coli* filtrates into guinea pigs even when the dose represents many multiples, per body weight, of the dangerous or even fatal calf dose.

The administration of the filtrate subcutaneously is without visible effect Similarly, feeding large numbers of living bacilli produced no manifest disturbances.

In support of the hypothesis of a genetic relation between the group of *B. coli* and of paratyphoid, a similar but less severe effect was produced in a calf by the intravenous injection of a bouillon filtrate of a paratyphoid strain.

[3] Branham, S. E., *J. Infect. Dis.*, 1925, xxxvii, 291.

[4] Steinberg, B., and Ecker, E. E., *J. Exp. Med.*, 1926, xliii, 443.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1927, Vol. xlvi, No 1, pp 133-140.]

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

II. MUTATIONS AND THEIR IMMUNOLOGICAL SIGNIFICANCE.

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PLATES 6 AND 7.

(Received for publication, March 18, 1927.)

A prominent and early phenomenon presented by the colon types from the ileum in calf scours was the appearance of thin peripheral outgrowths from surface colonies on agar plates which represented a mutation of the colony nucleus. These outgrowths began to appear in the incubator within 2 days on plates from the original intestinal material. They also appeared regularly on plate cultures from later transfers. One of the conditions preventing their appearance is a crowding of the colonies. These must be at least 1½ to 2 cm. apart to furnish the favorable environment. They form at incubator and room temperature.

Reproductions of the mutants which have thus far been more thoroughly studied from one or more angles are shown in Figs. 1 to 4 which may serve in place of detailed descriptions. It will be noted that two modes of formation of the outgrowths are presented. In one (Nos. 1127, 1085, 1192) they appear as rounded lobes or wings on the margin of the mother colony. This is thick, opaque, whereas the outgrowths are thin, partly translucent. As the lobes expand and the entire colony becomes enclosed in a zone of lobes the inner opaque colony becomes jagged and shrunken and often like an irregular cross in outline. The second form is shown in Fig. 4. The thin outgrowths appear as minute webs in notches of the original colony. These notches increase in width and depth and, as a result, the original colony has an outer zone of finger-like projections with the mutant outgrowth stretched between them. The tendency to form wing-like

expansions was not confined to one race. The strains figured differ from one another in one or more characters. Thus No. 223 is a motile, saccharose-fermenting strain; Nos. 1192, 1127, and 1085 are non-motile, non-saccharose-fermenting strains. All differ from one another in degrees of virulence.

The mutants obtainable on agar plates from lateral expansions of the colonies will be called (b) in the following pages, and the original nucleus of the colony (a). This mode of designation leaves open the use of other letters for mutants obtained in other ways. Only the (a) and (b) types will be considered in this paper.

Although morphological differences between (a) and (b) forms have not been observed, a definite distinction exists due to the presence or absence of an optically distinct capsule associated with a viscid condition of the growth in solid and in fluid media. The capsule was found only on (a) forms. Focusing on the periphery of the hanging drop of a diluted agar or a bouillon culture, one can frequently see the capsule, or a distinct spacing of the rods as they crowd together in the border of the drop. This spacing disappears as the marginal mass of bacteria begins to dry. The viscidity may be roughly estimated by the length of the thread which can be lifted up from agar or bouillon cultures after 1 or 2 days. The loss of viscidity and capsule occurred in all (b) forms studied. The presence of capsular substance was signalized by a heavy, glistening growth layer on sloped agar. This in some strains led to a slow sliding down of the growth from colonies on the slope, producing broad, vertical streaks Such appearances may be seen in cultures of *B. lactis aerogenes* and some strains of the Friedlander bacillus. In (b) types, the growth was thinner, partly translucent, and easily distinguished on sloped agar from the (a) types. It did not form descending streaks of growth from colonies.

In bouillon after 1 or 2 days, (a) forms produced degrees of viscidity, evident, if not by the formation of cobweb-like threads at the end of platinum loop or wire, at least by the tardiness with which air bubbles rose to the surface when the fluid was shaken These features were absent in (b) cultures Fermentation reactions were not changed by the mutation, nor was there loss or gain of motility.

Immunological Characters.—Marked differences in the relation of (a) and (b) forms towards animal hosts were in evidence in all strains

studied. (a) forms are agglutinated only in very low dilution in the sera of animals treated with them or else not at all. This difference in agglutinability was first observed in rabbits immunized with killed cultures. Later rabbits were treated with both (a) and (b) types. In one experiment (a) serum agglutinated the (a) form in a dilution of 1:40, the (b) form in a dilution of 1:40,960. A rabbit treated with the (b) form produced a serum which agglutinated the homologous form at 1:40,960, but did not influence the (a) form at all.

To obtain sera from larger animals, the cow, being the normal host of the types of *B. coli* under investigation, was chosen. The first cow (1109) was treated with living 24 hour bouillon cultures of *B. coli* 1127a over a period of about 5 months. The injection was chiefly intravenous, the initial dose about 1 cc. The reaction was always fairly severe (1) so that the increase in dosage was very gradual. The final dose was 17 cc. Agglutination of the (a) form was barely indicated at 1:20, whereas the limit of agglutination of the (b) form was 1:1,280. A second cow was treated in a similar way with living cultures of 1192a. After 6 months of treatment with weekly injections the dose safely endured was 10 cc. Agglutination tests with this serum were negative towards the homologous culture at a dilution of 1:10. Towards the (b) form, however, clumping was nearly complete at 1:640, the highest dilution tried. The (b) form was clumped in high dilution by the immune cow serum of Strain 1127a and this serum agglutinated the (b) form of 1192. Serum of a normal horse caused complete clumping at 1:640, the highest dilution tried. Serum of a normal cow clumped completely at 1:80 (b) strains of both 1192 and 1127. Serum of a normal rabbit, however, failed to clump at 1:10 Serum of a normal calf (1242) showed slight clumping of (b) in a 1:40 dilution. The mutation process had so changed the (a) form that it became agglutinable in high dilutions not only in homologous immune serum but also in sera of untreated cow and horse. Cross-agglutination with (b) forms of *B. coli* strains showing wide differences in virulence towards guinea pigs was observed. The agglutinins in horse and cow blood may have been due to the accumulation of antibodies resulting from intestinal multiplication of various types of *B. coli*.

To determine the relation of leucocytes to the (a) and (b) forms the

blood of young calves was tested. The Wright technique was used and the capillary pipette containing the mixture of blood and bacteria incubated for 10 minutes at 37°C. The phagocytic index was depressed by the use of citrate, and was highest when defibrinated blood was substituted. Although the results were irregular there was no exception to the findings that the calf polymorphs were nearly inactive in the presence of the (a) type, but the (b) type was readily taken up. The presence of agglutinins in high dilution in the immune rabbit serum towards (b) organisms interfered with its use in the phagocytic test.

Virulence of (a) and (b) Forms.—Early in 1925 a study of the virulence of living cultures of various strains of *B. coli* from scours towards

TABLE I.

Virulence of the Original and the Mutant Form Compared.

Strain of *B coli*	Minimum fatal dose		$\frac{(b)}{(a)}$
	cc	*cc*	
	(a)	(b)	
1085	0 04	1 0	25 0
1127	0 2	0 7	3 5
1192	0 04	1 0	25 0
223	0 06	0 7	11 6

guinea pigs was begun. The intraperitoneal route was chosen since subcutaneous injections failed to furnish any measurable data. The injection of the minimum fatal dose causes death within 24 hours. Very rarely an animal dies during the 2nd day. The reaction thus simulates the effect of a soluble or exotoxin. A comparative study of the minimum fatal dose of 24 hour bouillon cultures of (a) and (b) types yielded results shown in Table I. The last column expresses the relation between the minimum fatal doses of (a) and (b) forms. It will be noted that in these strains the minimum fatal doses of the (b) forms differ but little from one another, whereas the (a) forms show a greater spread.

Virulence in (a) types remained relatively constant when growths on sloped agar were transferred once in 4 to 6 weeks, incubated over-

night, and placed at 38–40°F. One strain (223) kept for 8 years in the manner described was still highly virulent for guinea pigs. In harmony with this persistence of virulence mutants did not appear in stock cultures kept in the manner described even after years of cultivation as repeated platings have shown.

The toxin production of (a) and (b) types was tested on calves as described (1). Two strains were used, Nos. 1069 and 1192. Given the same dose intravenously the characteristic respiratory symptoms were produced with equal severity by (a) and (b) filtrates of these two strains.

The uniform tendency of *B. coli* during mutation to lose certain characters indicating virulence and to acquire others also indicating loss of virulence marks this process as one of degeneracy but with reference to parasitism only. The degraded form persisted as such in cultures indefinitely. Two attempts were made to either restore or at least to modify it. One strain (1192b) was passed through a series of nine guinea pigs. Some of these died, others were chloroformed in 48 hours. Cultures were made from the peritoneal cavity and injected into the next of the series when 24 hours old. No increase in virulence or reappearance of viscid or capsular state was observed at the end of the series. A second attempt was made to change the mutant by passing it twice a day through bouillon tubes until more than 50 transfers had been made. At the end no changes towards the (a) form were manifest (2). The possibility of bringing the mutant back is not exhausted by these procedures. Not until such strains have been passed through the digestive tract of calves where the increased virulence of the (a) form may have been developed originally may we regard the change as irreversible.

DISCUSSION.

An examination of the voluminous literature on *B. coli* brings out certain data anticipating those briefly described. Baerthlein (3) in his extensive study of mutations of various species of bacteria mentions one group of paratyphoid strains, including two *suipestifer* and one *psittacosis* strain, which mutated on agar by sending out after several days a thinner, more translucent zone. In most writings the actual development of mutants from the original colony was not seen.

We are left to infer that cultures kept under certain conditions for a given period when plated presented two and sometimes more colony types. It is highly probable that the mutational changes went on in cultures kept in room temperature, exposed to diffuse light, and slowly drying out, although this is not stated. Massini (4) who was the first to observe mutations obtained his mutants from colony outgrowths resembling knobs (*Knopfe*). No reference was found to the loss of capsules, or the disappearance of a viscid product in mutant forms.

Gratia (5) isolated ten mutants from a single original stock of *B. coli*. These differed from one another in motility and mucoid growth. The mucoid condition in his strain was a mutational state and not a property of the original strain as in our *B. coli* types. Changes in morphology for the cholera vibrio, typhoid, paratyphoid, dysentery, and colon bacilli are described by Baerthlein (3). One form appeared plump, the other slender.

Modification of agglutinability due to mutation was observed by Gratia in his colon strain and by Baerthlein in *B. enteriditis*. His (b) forms were agglutinated more easily than (a) forms. The (b) agglutinins were absorbed with great difficulty, if at all. In a *B. coli* mutant, the agglutination presented much the same peculiar conditions as we have described them for (a) and (b) forms. According to Baerthlein no changes in agglutinability were noted in the cholera vibrio and in typhoid and dysentery bacilli. De Kruif (6) noted changes in acid agglutination but not when immune serum was used (7) in his studies on dissociation in the rabbit septicemia organism. Reimann (8), in studies of pneumococci, finds the S or original form as type-specific, the R or mutant form as group-specific. The former is agglutinable only in its type serum; the latter in its own and in S serum. The distinction of type and group agglutinin (Reimann) was possibly foreshadowed by Burk (9) who found *B. coli* races from the human intestine agglutinated by sera from a variety of normal animals including rabbits. Mutation as interpreted by Baerthlein is more or less temporary and forms may pass back from mutant to original type. He does not touch upon virulence. Later studies by others indicate that mutation, in pathogenic forms at least, signifies a degradation with reference to virulence or parasitism. This view is taken by De Kruif

and Reimann and is clearly indicated in our results with *B. coli.* Recovery of the original level of virulence did not occur in cultures and not in passages through animals. As stated above a reversion to the (a) type in the original host animals is not negatived by experiments made thus far. Concerning changes in the character or concentration of toxins no statements have been found.

This brief and incomplete review suggests that the term mutation has been used to designate a variety of changes which bacteria may undergo during artificial cultivation Some are obviously degradations and involve permanent loss of certain functions. Some are probably exaggerations or depressions of functions which can be brought back by certain procedures, as for instance the change back from R to S type by rapid transfers in bouillon (2).

The task of the future will be to redefine and classify the changes or mutations and evaluate them from different points of view. Especially desirable is a renewed study of those mutations in which definite functions are acquired, such as motility, fermentation of lactose (4), and of saccharose (10). These changes may be regarded as depressions or exaggerations or inhibitions rather than as new acquisitions until more exacting methods have been applied in a study of them.

GENERAL CONCLUSIONS.

On agar plates certain strains of *B. coli* from the ileum of calves suffering from diarrhea or scours promptly mutate and give rise to forms which have lost capsular substance, whose virulence has been greatly reduced, and which have gained very greatly in agglutinability and in being taken up by leucocytes. The original characters are not regained in cultures kept in the cold after development, nor in rapid transfers in bouillon, nor in passages through the peritoneal cavity of guinea pigs. Filtrates of 48 hour bouillon cultures contain as much toxin in the (b) as in the (a) form indicating no loss in this function.

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EXPLANATION OF PLATES.

Plate 6.

Fig. 1. Agar plate of *B. coli* 1127a. After several days incubation the outgrowths were nearly of maximum size and continued to expand but slightly in room temperature under a darkened bell glass. The plate was photographed after 21 days.

Fig. 2. Agar plate of *B. coli* 1085a. Details as for Fig. 1.

Plate 7.

Fig. 3. Agar plate of *B. coli* 1192a. Details as for Fig. 1.

Fig. 4. Agar plate of *B. coli* 223. On this plate a different form of outgrowth is illustrated. The mutant appears as a web-like expansion between clefts formed in the original colony.

All four plate cultures were prepared and photographed at the same time.

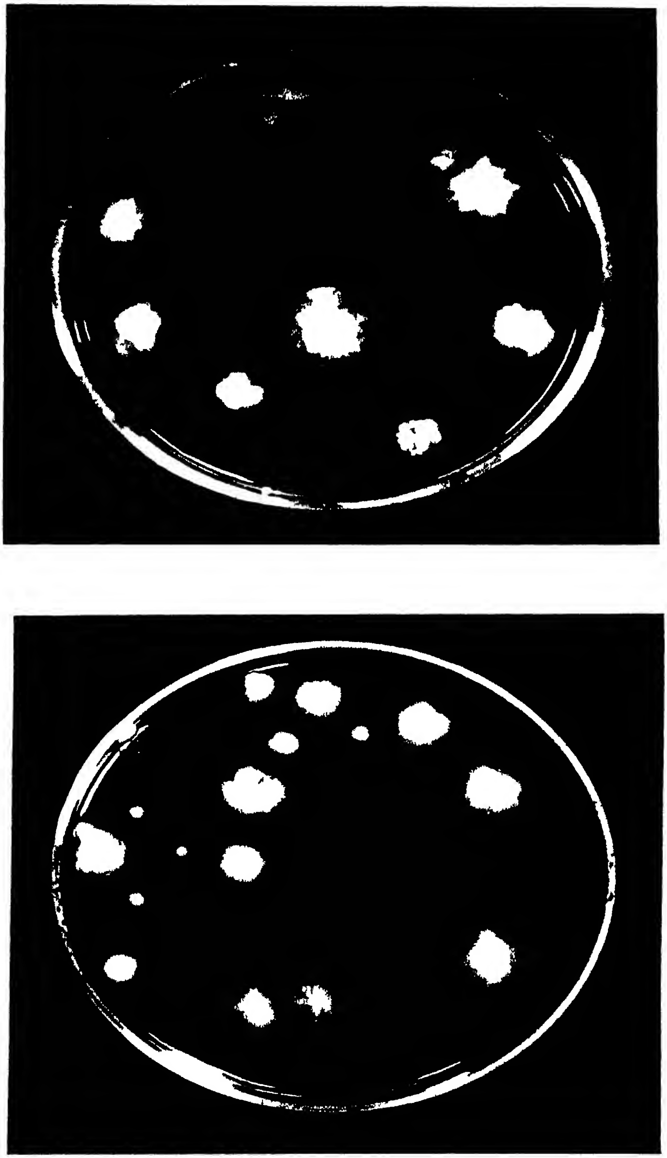

FIG 2.

(Smith and Bryant Pathogenic *B coli* from bovine sources II)

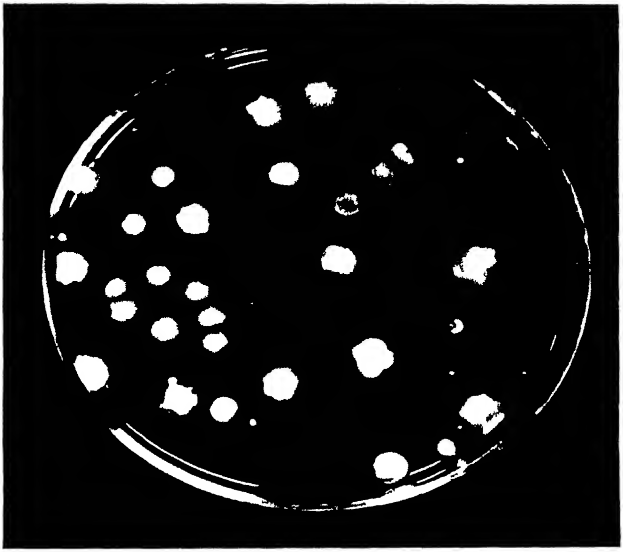

FIG. 3

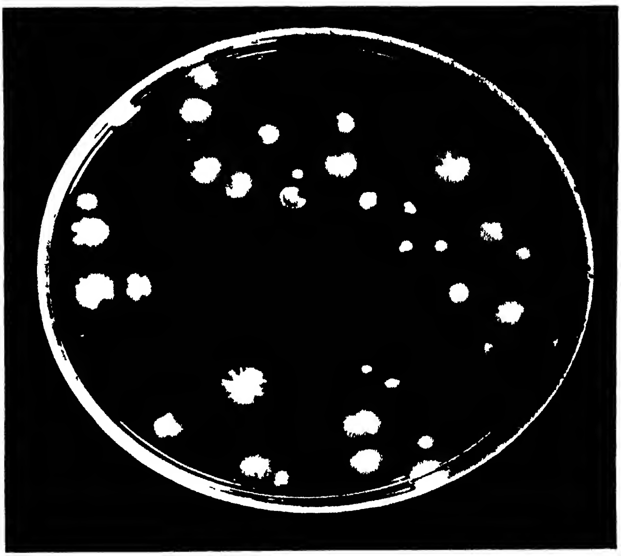

FIG. 4.

(Smith and Bryant: Pathogenic *B. coli* from bovine sources. II.)

[Reprinted from The Journal of Experimental Medicine, July 1, 1927, Vol. xlvi, No. 1, pp 141–154.]

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

III. Normal and Serologically Induced Resistance to B. coli and Its Mutant.

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(Received for publication, March 18, 1927.)

In view of the formidable differences in serological behavior of the original and the dissociated or mutant forms of *B. coli* described as appearing within 48 hours on agar plates made directly from intestinal contents,[1,2] it became desirable to analyze the virulence of the original and the mutant strain with the use of immune sera. To do this it became necessary to use the guinea pig in place of the cow or calf. This animal has been used in the past by various workers beginning with the early studies of the cholera vibrio by R. Pfeiffer. The intraperitoneal injection was chosen since the subcutaneous route fails to elicit any measurable reaction. To determine the minimum fatal dose bouillon cultures just 24 hours old were used. In these the turbidity of different strains and of the same strain in different lots of bouillon was markedly uniform and any more elaborate measure of dosage of the bacteria was dispensed with. The plating of definite dilutions of 24 hour bouillon cultures of the strain used chiefly in the experiments to be reported showed that there were present in 1 cc. 900 million of *B. coli* 1192a and 1 billion of the mutant (b).

The disease in guinea pigs following the intraperitoneal injection of the highly virulent or (a) form[2] was characterized by a prompt offensive and defensive reaction leading to death within 24 hours, or else only quiescence for 12 to 18 hours, loss in weight, and recovery

[1] Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, xlvi, 123.
[2] Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlvi, 133.

within 2 or 3 days. Very rarely an animal died after 18 to 24 hours. The minimum lethal dose may be titrated with nearly the same accuracy as is possible with diphtheria toxin. The effect of non-lethal doses may be roughly gauged by loss in weight. In early deaths, occurring between 6 and 10 hours, there is a trace of bloody fluid in the peritoneal cavity and indications of hemolysis. Certain viscera lying against the dorsal wall, such as the uterine horns and dorsal wall of cecum, may be spotted with hemorrhagic areas. Bacteria coat the peritoneum in large numbers. In animals dying between 12 and 24 hours, there is less congestion and hemorrhage and instead a beginning filmy exudate on liver and spleen. Bacteria may be very numerous or absent in films. All gradations are found between these extremes.

With the appearance of exudates, the viscera become covered with a translucent film of cells, not noticed at first until cover-slips are laid on the peritoneum and drawn away. Polymorphs are the only cell form at first and they increase in numbers with prolongation of life. When death occurs, in about 24 hours, there may be $\frac{1}{2}$ to 1 cc. of a ropy, opalescent fluid made up of leucocytes and bacteria.

In surviving guinea pigs, macrophages appear near the end of the first 24 hours and gradually increase in numbers so as to represent from one-fifth to one-half the number of cells present. Bacteria are very scarce at this stage. The cell exudate may be traced for 3 or 4 days, after which period it disappears. Occasionally whitish, viscid masses, 2 to 3 mm. in diameter, made up of polymorphs and macrophages remain in the folds of the omentum and spaces between the viscera. The lungs, at first normal in appearance, become greatly congested if death occurs as late as 18 to 24 hours. Usually from $\frac{1}{2}$ to 2 cc. of clear fluid is present in the pleural cavity at this time. Recovered guinea pigs, killed in 2 or more weeks, frequently have the spleen bound down by one or more firm adhesions to the abdominal wall.

The normal guinea pig weighing 350 to 400 gm. is capable of destroying a certain number of *B. coli* introduced into the peritoneal cavity. Among the strains isolated from calves the minimum fatal dose varied from 0.02 cc. to 0.5 cc. of a 24 hour bouillon culture. As a rule, three-fourths of this dose was still overcome by the guinea

pig. There was, however, a considerable fall in weight—from 40 to 50 gm.—when doses near the limit were injected, with a recovery of the original weight in 7 to 10 days. The injection of living *B. coli* thus gives almost as sharp a death point as is obtainable with diphtheria toxin. Of those that die, death occurs within 24 hours in fully 95 per cent. The bulk of the bacteria injected disappear from films within 24 hours, even in the rare cases that die after this time. In cultures from such animals made with loops rubbed over the viscera or dipped into traces of fluid present, usually a few colonies appear. In guinea pigs killed 2 or 3 days after inoculation, bacteria may frequently be cultured from the small clumps of cellular exudate filling interstices between viscera. After 4 or 5 days, these also are sterile.

Phagocytosis by polymorphs is evident between 12 to 24 hours, even in fatal cases, and increases with time. It is more pronounced following the injection of less virulent races. By the time macrophages appear, bacteria are scarce or absent. These cells are seen containing bacteria in very rare instances. In view of the usual function of macrophages in this experimental disease—ingesting polymorphs— the contained bacteria are probably within polymorphs in the macrophages. This is indicated when neutral red is used as a vital stain since the latter brings out the polymorph inclusions.

When a fraction of the fatal dose of a (b) mutant[2] of low virulence is given, the process followed by killing the animals at intervals indicates a rapid destruction of bacteria. When one-fourth the fatal dose is injected, representing about 0.25 cc. of culture fluid and equivalent to perhaps six times the number of bacteria in the fatal dose of the original or (a) strain, the number of living bacteria in the abdominal exudate falls to one-third within an hour. A small number may survive for 2 or 3 days in the lumps of polymorphs attached to omentum or viscera. The movement of polymorphs into the peritoneal cavity is more rapid in the presence of mutants and within 4 hours the peritoneal fluid is clouded with clumps of such cells. The cellular reaction beyond being more prompt does not differ in its further behavior from that already described for the (a) strain.

The toxicity, which maintains itself quite uniform in the same lot

of bouillon, must be retested when new lots of bouillon are used. If meat (veal) from young animals of nearly the same age is used the change from lot to lot is slight. If, however, meat from yearlings or older cows is used, the result may be quite different. In the case of Strain 1192a, the minimum fatal dose fell from 0.05 cc. to 0.02 cc. when meat from an older animal was used. Table I gives the results of one of several tests between the latter and a fresh lot of veal bouillon.

The Action of Immune Sera in the Protection of Guinea Pigs.—To study the factors involved in the varying behavior of *B. coli* in the peritoneal cavity of the guinea pig, immune sera were introduced into the problem. To obtain a fairly active serum cows were immunized. In what follows the sera of three cows were used. A

TABLE I.

Toxicity of Cultures Made from Different Lots of Meat.

No. of guinea pig	Weight	Dose (*B coli*)	No of bouillon	Results
	gm.	*cc*		
1	375	0 02	2252b (veal)	Lives
2	380	0 024	2252b "	"
3	375	0 02	2239 (beef)	Dead in 11 hrs.
4	380	0 024	2239 "	" " 11 "

brief statement of two of these will be found in an earlier publication.[2] No. 1109 was treated intravenously with living 24 hour bouillon cultures of *B. coli* 1127a. No. 1231 received intravenous doses of *B. coli* 1192a, and No. 1232, only Berkefeld filtrates of 48 hour bouillon cultures of 1192a. A more complete history of these cows will be given in another publication.

The sera thus produced were tested on guinea pigs.[3] The minimum fatal dose of a 24 hour bouillon culture of 1127a injected into the peritoneal cavity of a guinea pig weighing 350 to 400 gm. was 0.2 cc. Of the homologous immune serum, 0.1 cc. mixed with the culture was the minimum life-saving dose.

[3] The method here employed of injecting a mixture of culture and serum into the peritoneal cavity of the guinea pig had been used by Grosso in testing scours sera. See Grosso, G., *Z. Infektionskrankh. Haustiere*, 1912, xii, 54.

The marked protective action of colostrum and of cow serum administered to new-born calves suggested the idea that normal cow serum might in itself neutralize the fatal effect of *B. coli* in the guinea pig. In Table II is shown a comparative test with specific immune serum (*B. coli* 1127a) and normal cow serum. The immune serum protected against the minimum fatal dose in 0.1 cc. but not in 0.05 cc. amounts. The normal serum failed to protect in 1 cc. doses and a later test with 1.5 and 2 cc. did not retard death. Similar tests with sera from four other cows in doses of 1 to 2 cc. failed to retard death. In these different tests, the sera had been stored for some time and any toxic effect of the 2 cc. doses was not noticed.

TABLE II.

Effects of Normal Serum (Cow 1100) and Immune Serum (Cow 1109).

Weight of guinea pig	24 hr bouillon culture	Serum	Source of serum	Remarks
gm.	*cc*	*cc*		
450	0.2	—	—	Dead in 11 hrs.
460	0.2	0.1	Cow 1100	" " 12 "
465	0 2	0 2	" 1100	" " 11 "
440	0.2	0 1	" 1109	Lives
430	0 2	0 2	" 1109	"
525	0 2	0 05	" 1109	Dead in 12 hrs.
550	0 2	0 1	" 1109	Lives
510	0 2	0 2	" 1109	"
410	0 2	0 5	" 1100	Dead in 12 hrs.
425	0 2	0 7	" 1100	" " 8 "
440	0 2	1 0	" 1100	" " 12 "
490	0 2	—	—	" " 12 "

Strain 1192a was fatal to guinea pigs of the same weight in doses of 0.05 cc. In a preliminary test the serum of the cow treated with living bacteria saved life in 0.5 cc. doses. The "filtrate" serum failed to do this in the same dose. Later tests demonstrated that 0.05 cc. of the living-culture serum protected against 0.06 cc. culture, a trifle more than the minimum fatal dose. Table III illustrates the action of the immune serum at this stage.

A continuation of the treatment of both cows resulted in stronger immune sera. That of the animal treated with intravenous doses of

living cultures 11 months protected guinea pigs in doses of 0.005 cc. against $1\frac{1}{4}$ times the surely fatal dose. The serum of the cow treated with filtrates failed to do this in doses less than 0.5 cc. The law of multiples effective with diphtheria toxin and antitoxin mixtures fails when applied to the pathogenic action of living cultures of *B. coli*. In several trials, after the minimum serum dose effective in neutralizing $1\frac{1}{4}$ times the minimum fatal dose of culture fluid had been established as 0.005 cc. serum protecting against 0.025 cc. culture, a dose of 0.2 cc. serum, or 40 times the above dose, protected against

TABLE III.

Effects of Immune Cow Sera 1231 (Living Culture) and 1232 (48 Hour Culture Filtrate), Strain 1192a.

Weight of guinea pig	Dose of culture	Dose of serum	Result
gm	*cc*	*cc*	
350	0 04	—	Lives
355	0 05	—	Dead in 15 hrs.
370	0 06	—	" " 10 "
350	0.05	0.5, No. 1231	Lives
355	0 06	0.5, " 1231	"
370	0 075	0.5, " 1231	"
340	0 06	—	Dead in 5 hrs.
340	0 06	0.2, No. 1231	Lives
340	0 06	0.3, " 1231	"
350	0.06	0.1, " 1231	"
375	0 06	0.05, " 1231	"
350	0.06	0.5, " 1232	Dead in 8 hrs.
340	0 06	1.0, " 1232	" " 8 "

0.1 cc. culture, or 4 times the fatal dose. 0.1 cc. serum did not protect. Assuming in the latter case the protective dose to be actually 0.15 cc. serum, 4 times the minimum fatal dose requires 30 times the minimum serum dose.[4]

Although the immune cow sera showed a very definite protective action on guinea pigs, they failed to neutralize the toxic action of

[4] If we regard 0 02 cc. culture as controlled by the natural resistance of the guinea pig and subtract this from the culture doses used, we still have a culture ratio of $\frac{08}{0.005}$, or 16, to a serum ratio of $\frac{0\ 15}{0.005}$, or 30.

filtrates on calves. The sera were tested on fifteen calves. The dose of filtrate was 2 cc., that of the sera from 2 to 6 cc.

The respiratory symptoms already described[1] as produced by 2 day filtrates of both (a) and (b) forms of *B. coli*, were not neutralized or modified to any extent when mixed with either "living culture" or "filtrate" serum before injection. Thus the dose of filtrate usually injected, when mixed with 2 cc. and 3 cc. homologous "living" serum (1127a) failed to limit appreciably the violent reaction. The

TABLE IV.

Effect of Cow Serum (B. coli 1192a) on Pathogenic Action of Four Other Strains.

Weight of guinea pig	Strain	Culture dose	Serum dose	Result
gm		*cc*	*cc.*	
365	223a	0.07	—	Dead in 14–16 hrs.
360	223a	0 06	—	" " 24 "
365	223a	0 07	0 5	Lives
360	1085a	0 04	—	"
385	1085a	0 05	—	Dead in 14–16 hrs.
365	1085a	0 06	0 5	" " 29 "
355	1092	0 08	—	" " 29 "
360	1092	0 1	—	" " 14–16 "
360	1092	0.1	0 5	" " 22 "
350	1228	0 05	—	Lives
360	1228	0 06	—	Dead in 40 hrs.
360	1228	0 07	0 5	Lives

same was true of Sera 1192a, prepared with living cultures and filtrate respectively. Thus 2 cc. filtrate of (a) type or (b) type mixed with 2 cc. undiluted, homologous immune cows' sera both "living" and "filtrate," failed to moderate the reaction. In one calf (1159) the addition of 6 cc. "filtrate" serum did not check a severe reaction. The lungs of this calf 2 days after injection were still focally hemorrhagic and generally congested. If any mitigation of the toxic effects is to be produced by immune sera, it obviously will require prolonged immunization and large doses of the immune sera.

Polyvalency of Immune Sera.—The serological relationship of the

different strains of *B. coli* isolated from the ileum during scours or in normal condition was determined by using immune cow serum When the minimum fatal dose was mixed with the serum a certain grouping was made possible In Table IV two strains were obviously modified in their pathogenic action by the serum In two others the same quantity was not sufficient to prevent death.

The overlapping of the protective action of sera from two cows treated with different strains is brought out in Table V. Where the

TABLE V.

Polyvalency of Immune Cow Serum.

Strain	Minimum fatal dose	Protective dose of serum in 0 5 cc or less		Culture character
		1127a	1192a	
	cc	*cc*	*cc*	
223a	0 06–0 07	>0 5	0 5 or less	Saccharose + (motile)
1069a	0 5	>0 5	—	
1085a	0 04	0 2–0 3	>0 5	
1085b	1.0	0 5	—	
1092	0 1	0 3–0 4	>0 5	Saccharose + (motile)
1127a	0 2	0 1	—	
1127b	0 7	0 03	—	
1179	0 4	>1 5	—	
1189	0 5	>1 5	—	
1192a	0 04	0 3	0 05	
1192b	1 0	—	0 1	
1197	0 5	0 5	—	
1208	0.15	0 3	—	
1228	0 05	0 5	0 5 or less	Saccharose +
1358a	0 08	—	0 5 " "	

amount of serum needed is indicated as higher than the doses tried, there is of course the possibility that the serum has no effect whatever Thus Strain 1179 probably belongs to an entirely different serological group. There is also the possibility that by prolonged treatment of the cow the serum might become protective in the doses used.

It is of interest to consider the relative neutralizing power of immune serum towards the (a) and (b) forms. In an early test of serum of Cow 1109 (*B. coli* 1127a), 0.1 cc. serum inhibited the surely

fatal dose of 0.2 cc. of the homologous strain. 0.03 cc. of the same serum inhibited the surely fatal dose of 0.7 cc. of the (b) mutant. A more detailed test was made with an immune serum of Cow 1231 (*B. coli* 1192a). The final tests are given in Table VI. Tests made with the same drawing of serum on the (a) form showed that the surely fatal dose of 0.04 cc. (1⅓ × M.F.D.) was inhibited by 0.005 cc. serum

There is thus very little difference in the protective power of the (a) serum on the surely fatal doses, 0.04 and 0.6 cc., of the (a) and (b) forms respectively.

Non-Specific or Natural Resistance in Guinea Pigs.—It has been stated that guinea pigs are capable of disposing of considerable numbers of colon bacteria even when of maximum virulence. This phenomenon may be ascribed to a natural immunity. It had been shown by R. Pfeiffer and Issaeff[5] that by preparing the abdominal cavity of guinea pigs by the injection of bouillon, salt solution, and the like, an increased resistance towards the cholera vibrio could be produced. The same is true when *B. coli* is used. When guinea pigs receive into the peritoneal cavity 2 cc. bouillon, the minimum fatal dose of *B. coli*, or even a larger dose, is readily borne when injected 2 days later. If the experiment is continued and the same dose injected 10 to 14 days later, the guinea pig may die. In other words, the animal has lost in resistance during this period even though the

TABLE VI.

Immune Cow Serum 1192a and Culture B coli 1192b

Date of test	Weight of guinea pig	Dose of culture	Dose of serum	Result
	gm	*cc*	*cc*	
Feb. 4	370	0 5	—	Dead in 12 hrs.
" 4	360	0 6	—	" " 12 "
" 9	350	0 6	—	" " 15–16 "
" 9	355	0 6	0 005	Lives
" 9	330	0 6	0 02	"
" 11	345	0 6	—	Dead in 13 hrs.
" 11	355	0 6	0 0025	Lives

[5] Pfeiffer, R., and Issaeff, *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 355.

loss in weight following the injection of the first fatal dose has been amply compensated. Repeated trials were made to eliminate any possible errors in the tests. All showed that at least a certain number of guinea pigs had not recovered their normal resistance. In Table VII a final test is given. Eight guinea pigs received 2 cc. bouillon into the peritoneal cavity. 2 days later their weights had all increased. Three of them received 0.02 cc., and three, 0.025 cc. culture fluid. 2 days previous 0.022 cc. was found fatal. 11 days later all eight received doses from 0.016 cc. to 0.022 cc. in amount. Of these, two having received 0.02 cc. died within 10 hours It will

TABLE VII.

Original weight of guinea pig	All injected intraperitoneally, 2 cc of sterile bouillon				Result	Weight 9 days after 2nd inoculation
	Injected 2 days later		Injected 11 days later			
	Weight	Dose of *B coli* culture	Weight	Dose of *B coli* culture		
gm.	*gm*	*cc*	*gm*	*cc*		*gm*
385	—	—	440	0 02	Lives	400
355	—	—	450	0 022	"	430
375	395	0 02	420	0 016	"	415
370	400	0 02	430	0 02	Dead in 10 hrs	—
375	390	0 02	430	0 018	Lives	445
370	400	0 025	430	0 016	"	430
365	385	0 025	445	0 02	"	440
390	400	0 025	460	0 02	Dead in 10 hrs.	—

be noticed that 0.022 cc. did not kill a control, and that of the two that died, one had easily borne a dose one-fourth higher than the one which proved fatal. The other died following the same dose originally borne successfully. In this experiment all bouillon used for *B. coli* cultures was from the same lot.

Following an initial dose of *B. coli*, the recovered animals in a few instances have the spleen partly bound down by adhesions. The peritoneum recovers fully its original normal appearance. Permanent opacities and infiltrations are absent. The failure to resist the second dose does not appear to reside in any distinct anatomical defects or changes.

DISCUSSION.

In attempting to formulate the mechanisms by which the guinea pig is injured as well as protected we have several phases of the work to assist us. The study of the effect of the filtrate or toxin on calves shows us that most, if not all, of the pathological effect of the bacillus may be accounted for by its toxic filtrate. The extreme dilatation of the capillary system followed by hemorrhage as found in calves described also its action on guinea pigs, in spite of the fact that this species appears to be relatively indifferent to the culture filtrates. The guinea pigs either die within 24 hours or appear lively after 12 hours. Very few die on the 2nd day. The offensive weapon, then, of *B. coli* appears to be its soluble, diffusible toxins. Another fact which favors the view of the toxin action of *B. coli* is the difference in virulence due to different lots of bouillon. With *B. coli* 1192a the minimum fatal dose increased 2 to 2½ times from one lot to another.

The offensive action of the guinea pig probably is not a neutralization of this toxic filtrate, for in calves we find that a relatively large amount of immune serum mixed with filtrate does not relieve the symptoms due to the toxin. The guinea pig probably protects itself by some stored natural protective substance which, if present in sufficient quantity, checks the bacteria. If not, the bacteria continue to multiply and toxin production goes on until death ensues. This substance is mobilized by the injection of non-specific substances such as bouillon, so that a minimum fatal dose is easily borne. On the other hand, it appears to be used up by the injection of cultures and not fully restored even after 10 to 14 days, because at this time a second fatal dose or even a smaller dose may be fatal. If specific immunity consists in an overproduction of this substance it follows that resistance can be induced in guinea pigs only very gradually. It is highly probable that this substance destroys the bacteria by lysis, as is known for the cholera vibrio. The cooperation of polymorphs in this phase is highly doubtful. The cells appear too late on the scene. The macrophages come still later after the fate of the guinea pig has been settled.

To protect itself against this factor in the host, certain colon bacilli have developed a capsular substance. That this bacterial substance is primarily protective is suggested by its absence in mutants. They

retain the toxin-producing capacity, but lose, largely or wholly, the capsular, viscid substance and with it their specific resistance to agglutination and phagocytosis. Their virulence towards the guinea pig dropped in one culture to $\frac{1}{3}$, in several others to $\frac{1}{25}$ of the original dose. This change is completed a few days after isolation on agar plates. That death is still caused by the mutants is accounted for by the still normal toxin production. The large minimum fatal dose of living bacilli of mutant type is necessary since this type is readily destroyed and must be present in excess at the start.

The injection of living cultures of *B. coli* of the (a) or viscid type

TABLE VIII.

	Bacterial activities	Host activities
B. coli (a) type (M.F.D.. 0.04 cc. or 0.025)	Toxin ⟶ kills (offensive)	(Normal) antibody towards toxin, if any, increased very slowly or not at all by treatment
	Capsular substance (defensive)	⟵ (Normal) antibody greatly augmented by treatment (vaccination, immunization)
B. coli (b) type (M.F.D.· 1.0 cc. or 0.6)	Toxin ⟶ kills (offensive) Capsular substance (present in traces or absent) (defensive)	⟵ Unless host overpowered by toxin at the start, bacteria killed readily by antibodies

leads to the production of an immune serum which mixed with cultures and injected has a definite and potent protective action whereas normal cow serum has none in evidence in the technique used. When such normal serum is injected in quantities up to 2 cc., with cultures, death is not retarded. As stated above, the immune serum is not antitoxic. It probably cooperates with the normal forces of the guinea pig in suppressing multiplication and destroying the bacteria, since only a few survive in the peritoneal cavity after 24 hours. The interrelations of the several host-parasite activities, as suggested by the data given, are presented in Table VIII.

The presence of normal, antibacterial forces in the body and the production of highly specific antibodies by the repeated injection of

cultures have always been more or less incompatible factors in theories of immunity. The results reported in these papers offer the following hypothesis towards harmonizing these factors. The peritoneal cavity of the guinea pig and its walls (endothelium?) contain some substances normally which destroy the injected bacteria either by removing a protecting, viscid envelope and so exposing the organism to lysis, or by checking multiplication in one or more ways such as making the bacterial membrane more permeable to lytic factors or less permeable to nutritive elements. These substances are mobilized by the preliminary injection of bouillon. When some animal host is repeatedly treated with cultures, some one of these protective factors is highly developed in accordance with the capacities of the host. This gives the immune serum a one-sided, more or less specific effect, since the bacteria are suppressed in a certain way only.

According to this hypothesis, each host might furnish a serum of slightly different antibacterial activity towards the same microorganism according to the specific bacterial factor most easily handled by such host It is conceivable that if the toxin of *B coli* is antigenic, some host may protect itself chiefly by developing an antitoxin. The reason for serum specificity towards different biological groups of bacteria may be explained in a similar way, in that the bacteria themselves may present several widely different points of attack. Natural immunity according to this theory is simply a reservoir of numerous natural possibilities to be stimulated according to the different patterns presented by the microbes and the different capacities of the hosts for overdeveloping the individual contents of this reservoir. In particular the outer functional element, the ectoplasm, capsule, membrane, of the parasite, whatever it may be called, seems to stand out as the most important of the bacterial factors to be met by the host. If there is an active secretory function throwing off protective material, this, becoming diffused through the system of the host, may bring many cell territories to cooperate in increasing the output of protective antibodies. Immunity may come quickly and permanently, or else the microorganism gains the upper hand equally rapidly as in anthrax. The less the outer functional element is concerned in secreting and excreting protective substances, and the more it is concerned in transforming itself into a

locally protective envelope difficult to attack, the more the immunizing process becomes localized, and the slower the multiplication of the bacteria and the more difficult the acquisition of general immunity becomes.

The paratyphoid group, probably an offshoot of the colon group, may serve to illustrate this hypothesis. The paratyphoid bacillus produces a toxin which has similar effects on calves. It has no original capsular or viscid substance. None of the group yields any satisfactory antisera such as are readily produced by the frequently capsulated bipolar pneumococcus and colon groups. These invasive forms are however able to reach sensitive tissues and to produce their toxin in the close vicinity of highly sensitive cells. What this may mean is illustrated by *B. coli*. The toxins of this group introduced into the subcutis have little systemic effect. Brought in contact with the pulmonary epithelium artificially, as in intravenous injections, a very small amount produces profound effects.

CONCLUSION.

The interrelations between bacterial toxins, bacterial capsular (mucoid, viscid) substance, and certain normal protective factors in the guinea pig are studied with the aid of bacterial mutants and immune serum, and the results formulated in an hypothesis.

[Reprinted from The Journal of Experimental Medicine, July 1, 1927, Vol. xlvi, No. 1, pp. 155–166.]

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

IV. A Biochemical Study of the Capsular Substance.

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(Received for publication, March 18, 1927)

The carbohydrate gums which have been obtained from bacteria in sufficient quantity for chemical study are few in number The early workers were concerned with capsular material as such, and the possibility of its precipitation by homologous immune serum had not been thought of A bibliography and review of the literature are to be found in the papers of Heidelberger and Avery (1), or of Heidelberger (2).

Preisz (3) studied in detail the rôle of capsule and capsular material in infectious disease. He made preparations of capsular material in considerable amount. He believed it to be a mucin and stated that it gave no reducing sugar reaction. Whether the substance was prepared from virulent or avirulent strains, it removed from sera the factors responsible for lysis or the killing of anthrax bacilli (page 399) But he did not consider this a specific reaction, because similar material from cultures of organisms resembling *B. anthracis* also removed bactericidal substances from antianthrax sera *in vitro*. He thought that in the last stages of infection in the rabbit, capsular substance was present in blood and exudate in solution, neutralizing the antibody. He maintained that there was throughout a parallelism between virulence and the possession of a capsule.

With observations from studies such as this two lines of evidence converged towards and merged into the present concept of a polysaccharide reacting specifically with homologous serum, responsible for type specificity, but not eliciting antibody formation, and in all probability connected with the possession of a capsule and virulence in certain cases.

1. In 1921 Zinsser (4) called attention to the immunological importance of the non-protein constituents of bacterial cells. This frac-

tion which reacts with specific serum antibodies was termed "residue" in order not to imply too accurate an idea of its chemical structure. He believed this to represent the so called "haptene" of Landsteiner (5), for Landsteiner in his studies upon chemical modifications of precipitable antigens foretold the probable existence of materials which would react with antibodies without themselves being capable of inducing antibody formation when injected into the animal body. These conditions Zinsser's "residue" fulfilled, and the suggestion was made that they represented the haptophore group split off from the antigen as a whole and molecularly too small to induce antibody formation. It was also stated that this "residue" was analogous to the substances previously found by Cole (6) and by Dochez and Avery (7) in the blood and urine of typhoid and pneumonia patients.

2. The mass of evidence presented in the fundamental papers of Avery and Heidelberger (2) and Heidelberger and Avery (2) leaves us without grounds for doubting that it is a protein-free preparation that reacts specifically with homologous precipitating antibody and is responsible for type specificity. Very marked chemical differences were found correlated with serological specificity.

In the work of Mueller (8) and of Heidelberger, Goebel, and Avery (9) it was suggested that the "residue" or soluble specific substance is either identical with or connected with the capsule. But a question very naturally arises, why then is it possible to obtain "residue" from such apparently uncapsulated organisms as the tubercle bacillus, the staphylococcus, the meningococcus, and the influenza bacillus (10), as well as from the capsulated types of pneumococcus and the Friedländer bacillus? Is the capsule due to an increased production of a substance always present in the bacterial cell, a substance capable of reacting with antibody, but not of eliciting its formation on injection? Such a substance might represent the "haptophore" group of Ehrlich, and meet the conditions of the "haptene" prophesied by Landsteiner. Another question that naturally arises is the relation between capsular substance and virulence. A brief survey of the literature at once shows us that the possession of a capsule is almost universally regarded as increasing the virulence of an organism. This might be brought about in two ways. In the first place the capsular substance might act as a morphological factor, not dissolving away from

the organism, and insulating it from antibody action. Secondly, it might protect the organism by reacting in solution, at some distance from the bacterial cell, with antibody.

EXPERIMENTAL.

The possession of a number of mutating cultures of *B. coli* described by Theobald Smith and associates (11–13) offered an opportunity to look for the answers to the above questions in the case of this one species. It should be possible to find out if more precipitable carbohydrate is obtainable from the capsulated than from the non-capsulated strain from equal areas of culture surface, what its probable relation to the greater virulence of the capsulated strain may be, as well as something about its chemical properties

A consideration of the literature leads us to expect that a capsule would increase the virulence of an organism. This is, therefore, a very unique opportunity to obtain a quantitative estimate of the extent to which it enhances virulence. We think we are justified in excluding other factors, because the organisms are so alike. One, called the (b) form, is derived as a mutant from a colony of the other, called (a). Precipitation in the culture filtrate of either one is caused by the serum of the other, as well as by its own serum, as will be shown later. Filtrates of bouillon cultures, 24 to 48 hours old, of both (a) and (b) forms when injected into calves exert marked toxicity (11), but guinea pigs are only slightly sensitive.

These cultures of *B. coli* also afford an opportunity to test out the suggestion that "residue" or soluble specific substance is either identical with, or connected with, the capsule; because, if a certain substance were found to be present in extracts of the capsulated bacillus, and either not present at all or present in very small amounts in similar extracts of the mutant, it would be good evidence that it was indeed the material of the capsule. Of course it does not prove that there are not small amounts of other substances present, such as mucin. In the case of an alkaline extract of an extremely viscid strain of Friedländer's bacillus previously studied by the writer, there was present a large amount of substance or substances containing very little nitrogen in addition to the specifically active carbohydrate.

But in the case of the capsule of the colon strain there was nothing similar, at least not in appreciable amount

In what follows, the special strain of *B. coli*, 1192a, and its mutant 1192b, studied by T. Smith (11–13) was used throughout. In preliminary experiments, in which an alkaline extract of the bacteria was centrifuged and the supernatant precipitated with 2 volumes of 95 per cent alcohol, the precipitate, in the case of the viscid strain, was found to show a white ring very promptly at a dilution of 1 part in 100,000 by weight, with homologous serum. The mutant, non-viscid strain yielded a much less active precipitate, which gave a similar test more slowly and only up to a dilution of 1:1000 by weight, with either serum. It would seem therefore that in one case there was about a hundred times as much precipitable substance as in the other, so it seemed worth while to try to isolate the substance.

Preparation of Soluble Specific or Capsular Substance.—In all about 90 ordinary tin pie plates, each covered with a tin plate of larger size, were poured and inoculated. The ordinary stock veal infusion agar kept on hand in the laboratory was used, with the addition of 0.1 per cent dextrose just before pouring. The agar surfaces were very liberally inoculated with a suspension from 24 hour agar slants in normal saline. After 2 days' incubation, the growth was removed in distilled water (14). In this condition it was still extremely viscous. The bacterial emulsion was diluted to a volume of 500 cc., treated with about 75 cc. of 10 per cent KOH, warmed to 70°, and kept at that temperature about 30 minutes. After neutralizing and making slightly alkaline to litmus, adding about 10 gm of sodium acetate and 150 cc of 95 per cent alcohol, a clear supernatant was obtained after centrifugation and the sediment of bacterial bodies rejected. The substance in the supernatant was precipitated on adding 95 per cent alcohol up to 1.2 volumes It was partially purified by repeated solution and precipitation with 1 2 volumes of 95 per cent alcohol, added drop by drop with mechanical stirring, in the presence of plenty of electrolyte in the form of sodium acetate, usually from solution distinctly alkaline to litmus Precipitation from acid solution was also employed. The discarded supernatant contained much orange-brown material and very little specific substance as determined by the precipitin test. Precipitation with alcohol was continued 5 to 7 times, usually until the supernatant was practically colorless. The final precipitate, which unlike similar Friedländer material dissolves readily in warm distilled water, was put through a Berkefeld filter, after sufficient dilution, if still turbid. After concentration to convenient volume and reprecipitation with alcohol, it was washed with alcohol in increasing concentration and dried *in vacuo* over sulfuric acid. The yield from 90 plates was somewhat over 2 3 gm. It was active with homologous serum when diluted to 1 part in 2 millions, using the Fornet-Müller ring test.

Description and Analysis.[1]—The biuret test was negative. There was no precipitate (in ½ per cent solutions) with tannic acid, phosphotungstic acid, Esbach's reagent, 5 per cent copper sulfate solution, 10 per cent uranyl acetate solution, or saturated barium hydroxide solution. There was no color with iodine. The Molisch test was strongly positive But with 10 per cent basic lead acetate there was heavy precipitation and with 10 per cent ferric chloride a fairly heavy precipitate soluble in excess of the reagent.

The white, fluffy powder is readily soluble in hot water. In even a 1 per cent solution it is viscous and opalescent, somewhat resembling starch. It is not stringy, like the original growth on agar. It is very readily hydrolyzed by boiling with acid, and the hydrolysate reduces Fehling's solution, showing that a reducing sugar is present. It gives a slight naphthoresorcinol test for glucuronic acid and a slight test with orcinol, indicating the presence of glucuronic acid, but too faint for a pentose. There is a faint turbidity on boiling with barium hydroxide which is not removed by the addition of hydrochloric acid, thus indicating the presence of a hydrolyzable sulfuric acid. Very likely this is due to an impurity.

The substance, on drying in an Abderhalden dryer at 100°, lost 11.34 per cent of water. The elementary analysis, which was very kindly done by Dr. Elek, was as follows:

Ash 9 24 per cent —
C = 42 26 per cent
H = 5 82 " "
S = 0 6 " "
N = 0 6 " " (my own figure, micro-Kjeldahl, on 10 mg. portion)

Calculated on ash-free basis —
C = 46 56 per cent
H = 6 41 " "

For $(C_6H_{10}O_5)_x$ —
C = 44 4 per cent
H = 6 2 " "

So the carbohydrate nature of the substance is evident.

1 gm. of powder was used in the preparation of an osazone. It was hydrolyzed by boiling in 10 cc. of 2 per cent hydrochloric acid

[1] The writer was greatly assisted by Dr. P. A. Levene in this portion of the work.

under return condenser. The reaction was carefully followed by observing the rotation and the reducing sugar present in the solution from time to time.

Rotation		Reducing power equivalent to glucose
Initial	0 05	—
After ½ hr.	0 06	—
" 1½ hrs.	0 06	800 mg. (about 80 per cent)
" 5½ "	0 08	785 "

Since the values in the above table showed no significant change, heating was stopped, and the hydrolysis mixture neutralized with sodium hydroxide till only slightly acid to litmus. It was filtered and concentrated under reduced pressure to 60 cc. To obtain a phenylosazone it was warmed on the water bath with 2 gm. of free phenylhydrazine dissolved in glacial acetic acid. After 45 minutes on the water bath, it was heated a trifle more and immediately filtered through a folded filter. To this filtrate a second time phenylhydrazine in glacial acetic acid, to the amount of 1 gm., was added and the solution kept on the water bath as long as an osazone was forming. This was again filtered off, and a third precipitate of osazone obtained from the filtrate after standing about 1 hour more. The first lot of osazone was kept separate, but the second and third, which had a similar appearance, were combined. Each precipitate was taken up in methyl alcohol to remove adhering impurities, so that the final osazones were free from oil drops and consisted of long crystalline needles or rosettes.

The melting point of the first was 196°C. The second sintered at 180° and decomposed at 195°. Neither showed optical rotation The second was analyzed for nitrogen, with the following results: From 0.0620 gm. substance was obtained 8.30 cc. nitrogen at 763.3 mm. Hg and 22°C. This is equivalent to 15.55 per cent nitrogen. (Calculated for $C_{18}H_{22}O_4N_4$, N = 15.63 per cent.)

The fact that the observed rotation of the hydrolysate was very low for the reducing power, and that the osazone seemed to be entirely inactive optically, makes it seem likely that the hydrolysis mixture contained at least two hexoses, one dextro- and the other levorotatory The osazones were not separable, or they may have been identical. From these facts it seems established that the substance obtained

from the colon bacillus belongs in the class of carbohydrates, as shown by the elementary analysis, that it contains glucuronic acid, and hexoses. The total reducing power of the sugars obtained on hydrolysis calculated as glucose was equivalent to 80 per cent of the weight of the substance. The substance thus differs from all the others reported.

Attempt to Relate the Specific Carbohydrate Obtained from the Capsulated Bacillus to Its Greater Virulence.—The question naturally arose[2] as to whether it were possible to find out how the capsular material acts to increase virulence. Whether it may function as a morphological capsule, or partly dissolve and thus act at a distance by neutralizing the antibody in the blood of the host.

TABLE I.

Guinea pig No	Weight	Culture	Extract	Result
	gm	*cc*	*mg*	
1	355	0 3	—	Lives
2	350	0 6	—	"
3	350	0 9	—	"
4	360	1 2	—	Dead in 22 hrs.
5	355	0 3	10	Lives
6	350	0 6	10	"
7	350	0 9	10	Dead in 10 hrs.

It seems, from a consideration of the following observations, that in the case of the viscid strain the substance must be acting to a large extent as a closely adherent protecting layer, probably permitting multiplication of the organisms before they can be destroyed. We know from the papers by Smith and associates (11–13) on these mutants that capsulated strains are practically not phagocyted at all. If capsular substance is of significance in the production of virulence it may very likely be in connection with non-phagocytability.

A few experiments were made on guinea pigs to see if the substance extracted from cultures as a carbohydrate had any influence on the course of the disease produced by *B. coli.* The mutant (b) was tried

[2] For suggestions at this point the writer is particularly indebted to Dr. Hans Zinsser and Dr. J. H. Mueller.

first. Table I illustrates the procedure for testing any increase in virulence due to admixture of the living 24 hour bouillon culture with the extract. This was ground up in normal saline. The injection was made into the peritoneal cavity of guinea pigs.

An apparent increase in virulence may be deduced from the table. Several other tests yielded similar data. The (a) form was tried next to see whether it also might be favored by the preparation. 5 mg. of the preparation in normal saline were mixed with graded amounts of the culture and the mixture injected into the peritoneal cavity. The minimum fatal dose was definitely lowered. When, as controls, 5

TABLE II.

1192a and 1192b grown 24 hrs in flasks containing 75 cc. of veal bouillon.

Serum of rabbit injected with strain	Capsulated strain, 1192a			Mutant, 1192b		
	Filtrate not diluted	Diluted 1/2	Diluted 1/4	Filtrate not diluted	Diluted 1/2	Diluted 1/4
(a)	+*	Tr.	0	+	+	Tr.
(b)	+	0 (?)	0	+	0 (?)	0
1192a and 1192b grown 48 hrs. in exactly similar conditions.						
(a)	+	?	0	+	+	Tr.
(b)	+	0	–	+	0	–
1192a and 1192b grown 3 days in exactly similar conditions.						
(a)	+	+	0	++	+	Tr.
(b)	+	+	0	++	+	0 (?)

* + means a ring that can be seen distinctly. None of these rings were heavy.

mg. of gum tragacanth, and a feebly turbid homogeneous suspension of aleuronat, probably less than 5 mg., were injected with *B. coli*, the same increase in virulence was observed.[3] This method of demonstrating the possible relation of the carbohydrate to virulence was not pursued any farther, since it was evident that some other method would have to be worked out, eliminating mere injury to the peritoneum. The protocols relating to the test with the (a) form are therefore omitted.

[3] See also Benians (15).

It is to be noted that the amount of precipitable substance found in filtrates of early cultures is actually very small, both in the (a) and the (b) forms. This is brought out in Table II.

The two rabbits yielding the highest titer serum, Nos. 1 and 2, had been given intraperitoneal injections, 5 days apart, of increasing amounts of killed growth from 24 hour agar slants emulsified in normal saline. It is impossible to clear the culture of the capsulated strain by centrifuging. An attempt was made to see if any rings could be distinguished in a dilution of 1:8 to eliminate turbidity, but there was not enough precipitable substance to give a reaction at this dilution. So recourse to filtration through a small Berkefeld was necessary. Saline was always passed through first and tested to insure absence of anything giving a ring with the most potent serum (a). Then half the 75 cc. of culture fluid was passed through and rejected, since precipitable substance might be adsorbed by the filter at first. Inasmuch as all the material of all preparations made had been put through Berkefeld V's or N's and there was no trouble with adsorption, the precaution of using only the last part of the filtrate would seem sufficient.

DISCUSSION.

The condition presented by *B. coli* (a) and (b) was quite different from that found by Dochez and Avery (7) when studying the pneumococcus. During the early stages of vigorous growth this organism forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the blood and urine. The writers could not demonstrate that this substance was responsible for the intoxication occurring in lobar pneumonia, but they were able to state, after the study of 112 cases, that if large amounts were excreted, the outcome was usually fatal. The table on page 479 of Dochez and Avery's paper is particularly instructive. This is from the study of a flask inoculated with an organism of Type III, which forms somewhat more precipitable substance than I or II. After only 4 hours, the undiluted filtrate showed heavy flocculation with homologous serum. A 24 hour culture showed a heavy precipitate at a dilution of 1:30. A trace was recorded at a dilution of

1:120. Evidently the material diffuses out into the culture fluid in considerable amounts.

When we consider the capsulated *B. coli*, we find an entirely different state of things. As shown in the table, there was not a trace of precipitin reaction visible in the case of a 24 hour culture at a dilution of 1:4, nor was there in that of a 3 day culture. When undiluted, there was a good, but not a particularly heavy reaction, even with a precipitating serum of very high titer. Evidently in this case the precipitable substance does not dissolve out into the medium during the growth period. The amount of specific carbohydrate capable of reacting with homologous serum in a 1 to 3 day culture of either (a) or (b) is practically nil.

There remains the question of the relative amounts of precipitable material present in the two strains, the one capsulated and the other not, for this ratio is perhaps at this time the best chemical evidence obtainable of a relation between capsule and soluble specific substance. More crude material is obtainable from equal areas, for example, the surface of ten pie plates in the case of the capsulated strain. No count of the relative numbers of organisms involved has been attempted, but from the uncapsulated strain only about two-thirds as much crude material can be obtained per unit of culture surface. And this material is about 100 times *less active* with high titer precipitating serum (from either strain) than is similar material from the capsulated strain. No attempt has been made to prepare or purify a large amount of the "residue" from the mutant.

The simplest assumption is therefore that suggested by Mueller (8) and Heidelberger, Goebel, and Avery (9), that capsular material and soluble specific substance are identical. In the case of a morphological capsule, the specifically precipitable substance or "residue" probably is produced in much larger quantity and located peripherally Other substances, such as bacterial mucins, may also take part in the capsule formation. In the case of the capsulated *B coli*, there was no indication of more than a trace of mucin. From the ease with which the material can be obtained in relatively pure condition, it is very unlikely that much is present. On the other hand, in the preparation of specific carbohydrates from a peculiarly viscid strain of Friedländer's bacillus (unpublished) there was obviously a *very*

large admixture of an impurity that was probably mucin, or something similar.

The substance obtained from the capsulated strain of the colon bacillus is not identical with any specific carbohydrate thus far already described. It is composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is slight. It is precipitable by basic lead acetate and ferric chloride. It is rather lighter in weight and more readily soluble in distilled water than are some similar substances prepared by the writer from the Friedländer bacillus. The presence of glucuronic acid is indicated. Some evidence for a relation between capsule and specific substance was obtained. From equivalent areas of growth on agar in the case of the mutant about two-thirds as much material, as in the case of the viscid strain, precipitable with 1 volume of alcohol, can be obtained. This material, obviously very impure, is about 100 times *less* active with homologous serum than similar material from the capsulated strain. Although this cannot be interpreted as proving that the capsulated organism contains 100 times as much specific carbohydrate as the mutant, it is a very significant difference.

The specific substance of the capsulated colon strain does not diffuse out into the fluid culture media, as does that of the pneumococcus. There is so little present in the filtrate of a 24 hour culture, such as used in the original virulence tests, that it indicates that the substance functions distinctly as a capsule, a protecting layer, rather than in solution. Attempts to recombine the extract with living bacilli in testing virulence proved unsatisfactory, since non-specific additions in minute amounts also reduced the resistance of the peritoneal cavity of the guinea pigs.

SUMMARY AND CONCLUSIONS.

1. The soluble specific substance obtained from a capsulated strain of *B. coli* is not identical with any specific substance heretofore described. It is a carbohydrate, composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is low. Glucuronic acid is probably present in the molecule.

2. Crude "residue" or specific substance obtained from the uncapsulated mutant was about 100 times *less* active with homologous serum than similar material from the capsulated strain. This supports the view that capsular substance and soluble specific substance are the same. In cases where there is a well marked capsule, the specific substance is probably produced in greater amount and located peripherally.

3. Capsular substance is probably significant for virulence when functioning as a morphological capsule. It is present in filtrates of young culture only in very small amounts.

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[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1927, Vol. x, No. 6, pp. 949–960.]

UDDER SIZE IN RELATION TO MILK SECRETION.

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(Accepted for publication, May 31, 1927.)

Our knowledge of the mammary gland as related to its size and function has been largely based on the observational relationship which exists between udder size and the milk the cow is able to give.[1] Briefly considered the data available consist of two measurements, the milk production of the cow in pounds and the relative degree of perfection of the udder in size, shape, and quality as measured on an arbitrary scale called her score. Study of this information on 1674 Jersey cattle shows a correlation between the size and quality of the udder and the milk yield which the cow was able to produce. This correlation, while markedly significant, is low, .19 ± .016. The evidence thus points to the conclusion that despite the obvious sources of error the size of the udder is a function at least of the milk which the cow is able to secrete. The just published work of Gaines and Sanmann[2] supports this hypothesis while the work of Maxwell and Rothera[3] and the opinions of many dairymen and veterinarians citing the size of the udder as being too small to hold the milk the

[1] Gowen, J. W., Conformation and its relation to milk-producing capacity in Jersey cattle, *J Dairy Sc.*, 1920, iii, 1. Studies on conformation in relation to milk-producing capacity in cattle. II. The personal equation of the cattle judge, *J. Dairy Sc.*, 1921, iv, 359. Studies on conformation in relation to milk-producing capacity in cattle. III. Conformation and milk yield in the light of the personal equation of the dairy cattle judge, *Annual Rep. Maine Agric. Exp. Station*, 1923, 69.

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cow is able to give at any one time controvert it.[4] Stated quantitatively the problems before us are: (*a*) what proportion of the milk given by the cow at milking is already stored in the udder; (*b*) what correlation exists between udder size and milk yield; (*c*) how much secreting tissue is necessary to manufacture a pound of milk; (*d*) what is the relation between secreting and supporting tissue in the udder.

For the work herein cited nine dairy cows were used. These cows were milked twice a day, the time of the first milking being 1.00 p m., and that of the second milking, 10.00 p m. The cows were milked on these hours for 5 days before they were killed. Their milk was weighed after each milking and a sample of the milk taken for the analysis of the lactose content. The cows were then shipped 10 miles to a slaughter house where they were killed at 1.00 p.m.[5] The technique was varied for the first two cows. The udder of one of these cows was minced and the minced material subjected to a pressure of 2000 pounds in an hydraulic press. This technique proved unfortunate in view of the fact that the udder material holds the milk secreted very tightly, it being almost impossible to press out any of the milk although it may be milked or drained out. In consequence the minced material was simply driven into the cloth and had to be extracted with water in the same manner as that used for the later seven cows. The second cow's udder was cut into small strips and allowed to drain and then these strips were put into the ton press. Here again it was impossible to press out any amount of secretion. The material was then extracted with water in the manner described for the other seven cows The results of this unfortunate technique showed clearly that the musculature of the udder is such that milk may be extracted from it far more easily by the ordinary methods of milking with the cooperation of the cow than is possible with relatively large pressures applied under external conditions The technique for the other seven cows consisted of milking these cows at 1.00 p.m. and 10 00 p.m. for 5 consecutive days, determining the milk flow and lactose percentage in the milk for each of these milkings The cows were then killed at exactly the hour of previous milking, the last three being killed after milking, the other four being killed with the udder full of milk. The udders were then carefully dissected off, cut in strips, and drained for the milk which would quickly flow out. The remaining material was then ground and extracted with water three different times, about

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[4] That this opinion lacks foundation in fact in at least five cows is shown by the work of Swett, W. W., Relation of conformation and anatomy of the dairy cow to her milk and butterfat-producing capacity. Udder capacity and milk secretion, *J. Dairy Sc.*, 1927, x, 1.

[5] It is a pleasure to acknowledge our indebtedness to Penley's Packing Company, Auburn, Maine, for their cordial cooperation.

50 pounds of water being used in each extraction, the fluid material being drained through cheese-cloth bags. The remaining extracted udder material was then dried, ground, and the little sugar which remained in it was determined by first removing the fat with gasoline and then extracting with water. Printing cost allows only publication of the totals.

Table I shows the milk production of the cows for the 1 o'clock milking and the 10 o'clock milking for the 3 days previous to their slaughtering.

TABLE I.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking, for the 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average milk yield	
	1 p m.	10 p. m
111	12.9	7.4
124	17 9	10 0
132	15 6	10 3
136	15.7	13.8
148	13.5	8 3
154	11.7	5.9

TABLE II.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average milk yield		
	1 p m	10 p m	Last 1 p m milking
97	4 7	2 2	2 8
114	4.1	2 8	3 6
153	10 8	6 9	9 6

Table II shows the milk production of the cows, at the 1 o'clock and 10 o'clock periods, which were slaughtered just following milking.

The milk productions in Table II are lower than those in Table I. The cows had to be shipped 10 miles to the slaughter house before they were killed, so that this disturbance probably played some part in their milk production before slaughter, tending to decreased secre-

tion and to retention of that which was secreted. This is noted in the fact that the milk production of Cow 97 was 1.9 pounds less than her 1 o'clock average for the 3 previous days. No. 114 had 0.5 of a pound less than the average of the 3 previous days, and No. 153, 1.2 pounds less than the average of her earlier milkings. It was found that 0.8 of a pound of milk could be drained out of the udder of No.

TABLE III.

Average Lactose Per Cent for 1 p.m. and 10 p.m. Milking for 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average lactose	
	1 p. m	10 p m
	per cent	*per cent*
111	4 62	4 60
124	4 78	4 72
132	5 28	5 33
136	5 05	5 10
148	4 81	4 89
154	4 84	4 90

TABLE IV.

Average Lactose Per Cent for 1 p.m. and 10 p.m Milkings of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average lactose		Lactose
	1 p m	10 p m	Last 1 p m.
	per cent	*per cent*	*per cent*
97	3 98	4 03	3 80
114	4 72	4 80	4.85
153	4 50	4 58	4.50

97, although the milking had been performed very carefully. Cows 114 and 153 showed some milk left in the udder, but not enough to weigh. This milk was allowed to go into the determination of the sugar content in the udder, following milking.

Tables III and IV show the percentages of lactose found in the daily milkings of the cows given in Tables I and II. It will be noticed that there appears to have been little or no change in the lactose per-

centages of Cows 97, 114, and 153 from the average for the milk of the 3 previous days. It may be concluded from this that any excitement incident to the trip to the slaughter house for these cows had no effect in changing the lactose content of their milk.

The total lactose for all of the extractions is shown in Table V. This total lactose divided by the average lactose percentage for the 1 o'clock milk yield of the 3 preceding days gives the amount of milk necessary to account for this total amount of lactose. Throughout, the materials added checked well with those extracted. The udder material remaining after extraction shows an average of less than .04 per cent lactose.

TABLE V.

Weight of Lactose Extracted from Udders and Milk Equivalent of Lactose, Pounds. August, 1926.

Cow No	Weight of total lactose	Milk equivalent
111	.718	15.5
124	.931	19.5
132	.774	14 6
136	798	15 6
148	614	12.8
154	538	11.2
97	247	6 2
114	140	3.0
153	150	3.3

The lactose extracted from the udder, in the case of the unmilked cows, represents the lactose contained in the milk which would be drawn on milking and the lactose which would remain in the udder. For the cows milked just before death the lactose represents the milk which could not be drawn from the udder by milking. This, converted into pounds of milk, represents for Cow 97, 6.2 pounds; for Cow 114, 3.0 pounds; for Cow 153, 3.3 pounds. The large amount of milk remaining in the udder of Cow 97 calls for comment. After the milking was completed and the udder dissected off about 3/4 of a pound of milk was found in this udder which could easily be drained out of it. This milk was held up by the cow even though the milking was performed carefully by an experienced milker. It will be noticed

that no such amount of milk was found in the udders of the other two cows, 114 and 153. In fact, not over .1 of a pound of easily drained milk was left in these udders. Should the 3/4 of a pound of free milk be left out of consideration there is still 5.4 pounds found in this udder. The total milk of Cow 97 is consequently much larger than her previous milk yield would lead one to expect. How this cow manufactured this extra 2 or 3 pounds of milk the authors do not know. These three cows' milk yields before killing, in comparison with the average of the 3 previous days, were 1.9 pounds less than would be expected for Cow 97, .5 of a pound less for Cow 114, and 1.2 pounds less for Cow 153. The reduction in milk yield seems to be accounted for by the strange conditions under which the milking took place and the nervous excitement previous to milking. If these values be subtracted from the milk found in the udder, determined as lactose, Cow 97 has 4.3 pounds of milk remaining in the udder; Cow 114, 2.5 pounds; and Cow 153, 2.1 pounds. Cows 114 and 153 appear to check nicely. Cow 97 has about 2 pounds more milk in the udder than would be expected on the basis of the other results. Whether the average of the three cows should be used or only the average of the last two is perhaps a question. If the average of the three cows is used it is found that the udder contains 3 pounds of milk when it is supposedly milked dry. The milk found in the udders of the six remaining cows determined as the lactose equivalent, represents the milk which would be drawn in normal milking plus that which was retained in the udder after the cow was considered dry. If these figures be compared with the amount of milk which the cows gave it will be noted that they correspond fairly well. Thus the average milk production of Cow 111 was 12.9 pounds while the amount accounted for is 15.5 pounds, that for Cow 124 was 17.9 pounds while the milk accounted for is 19.5 pounds, that for Cow 132 was 15.6 pounds while the milk accounted for is 14.6 pounds, for Cow 136 the milk production was 15.7 pounds while the milk accounted for is 15.6 pounds, for Cow 148 the milk production was 13.5 pounds while that accounted for was 12.8 pounds, and finally the milk production of Cow 154 was 11.7 pounds while that accounted for was 11.2 pounds. It will be noted throughout that the amount of milk accounted for and the amount of milk drawn from the udder correspond fairly

closely. They show, furthermore, the following relative relationships: the higher milking cows show the larger amount of milk in their udders; the lower milking cows, the lower amount of milk in their udders; with the medium yielding cows between the two extremes. The average milk production for the six cows was 14.6 pounds; the average amount of milk accounted for for these cows was 14.9 pounds. It is to be remembered that of this milk accounted for on the basis of lactose there is probably remaining in the udder after milking between 2 and 3 pounds of milk. Thus from the 14.9 pounds accounted for on the basis of lactose there should be subtracted between 2 and 3 pounds due to the amount of milk which it is impossible to milk from the udder. The comparison of the amount of milk accounted for should be therefore between 12 and 13 pounds as contrasted with the 14.6 obtained. All these results show clearly that at actual time of milking between 80 and 85 per cent of the milk can be accounted for in the udder of cows milking up to 30 pounds of milk a day.[6] The experiment therefore points to the conclusion that 20 per cent is a maximum and 10 to 15 per cent, a probable value for the amount of milk which may possibly be secreted in the udder during the time of milking.

This value is considerably less than that obtained by Maxwell and Rothera in their experimental work. Their results are largely dependent upon the accuracy with which the lactose in the milk of the cat is represented by their assumed value of 5.07 per cent. Gaines and Sanmann cite Folin, Denis, and Minot's analyses on 19 samples

[6] The technique of the experiment is such that it is necessary to make the sugar analysis over a period up to 4 days from the time of extracting the udder. The extracted materials were carefully preserved on ice and 10 drops of formalin added to each pint of extract This method of preservation has shown little change in the sugar content although that possibility is to be considered The acidity test was throughout all of the experimental work between 005 and 19 per cent. The milk acidity from the cows was throughout between .1 and .13 per cent. There is then a chance that some of the sugar in the material extracted from the udder was converted into acid before it could be read. Another and perhaps more serious chance to decrease the lactose accounted for in comparison with the milk of the 3 previous days is a reduced milk secretion on the day of killing incident to the trip to the slaughter house. Thus all the factors tend to reduce the milk accounted for in contrast to the average of the 3 previous milkings.

of the milk of three cats. These data give a range in percentage of lactose from 2.3 to 4.0. For 26 samples on four cats the average was 3.4. Should 3.4 be used in place of 5.07 as the lactose per cent of cats' milk, the total secretion of the mother cat as extracted by the kittens is accounted for.

Our data are in general accord with those of Gaines and Sanmann. Their technique on Cow 2 is, we believe, better than ours in that there is less chance of losing lactose in the manipulation of the udder and we are inclined to the view that our results show somewhat less lactose than was actually present in the udders.

The available information thus indicates that the mammary gland at time of milking contains the majority of the lactose to be secreted in milk.

Relation between the Size of the Mammary Gland and the Milk It Secretes.

The relation which exists between the size of a gland and the size of the product which it manufactures is an almost unstudied problem of gland physiology. In fact the problem has been approached only by judges of dairy cattle. These qualitative data have led to the assertion that the size and quality of the udder does to some extent indicate the productive capacity of the cow. The records of these nine cows in the experiments herein described furnish unique, fairly exact evidence on this problem. The correlation which can be derived from these data is admittedly open to a very large probable error. It does, however, furnish an important guide to the results which might be expected on more extensive data. The material also has the advantage that the determinations are quantitative and relatively accurate as contrasted with those heretofore used. Statistically considered the results as treated are significant since $r = .96$, $t = 9.0$ and P for $N = 9 < .01$.

The most interesting comparison is that between the weight of the udder with the contained milk and the milk production which the cow normally gave. This measurement is also the most exact so far as the collection of the data is concerned. Table VI shows the relation of the milk yield to the weight of the udder and contained milk.

Table VI and Fig. 1 show that the average weight of the udder and

its contained milk increases as the milk production which the cow is then giving increases. This increase amounts to about 1.2 pounds

TABLE VI.

Total Weight of Udder and Contained Milk Contrasted with the Cow's Average Milk Yield at the Same Period.

Cow No	Average milk yield	Weight of udder and contained milk
114	4 1	16 6
97	4.7	18 8
153	10.8	26 1
154	11 7	24 2
111	12 9	29 3
148	13.5	27.2
132	15 6	28 7
136	15 7	30 6
124	17 9	36 0

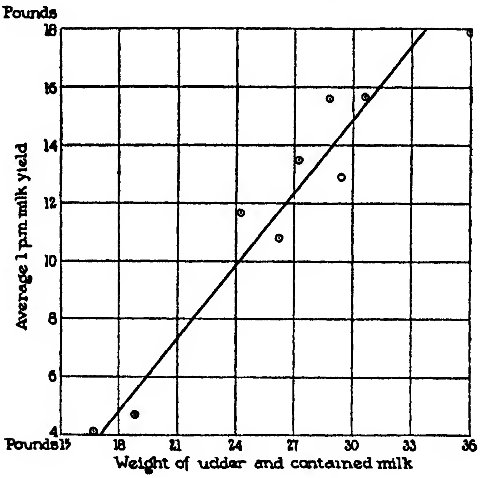

FIG. 1. Relation between average 1 p.m. milk yield and the weight of the udder and contained milk.

for the weight of the udder and its contained milk as the cow increases in milk production 1 pound, the increase being fairly regular over the

entire range. From this fact it may be concluded, tentatively, as the probable errors are large, that for creating a pound of milk between 10.00 p.m. and 1.00 p.m. (15 hours), .2 of a pound of secreting mammary tissue would be necessary. On the basis of these few observations the relationship between milk yield and the mammary gland size appears to be linear. In view of this fact, if the line of general relationship is extended to the point of no milk production, the udder would weigh about 12 pounds. On the basis of the results for Cows 97, 114, and 153 such an udder would still contain 2 to 3 pounds of milk. The mass of udder tissue in the practically dry cow would consequently be 9 to 10 pounds. This might be conceived of as connective tissue supporting the glandular structure, furnishing the surrounding tissue for the teats and alveola spaces.

The Remaining Uddcr Material.

As indicated earlier the udder with its contained milk was first cut and drained of the milk which would flow out of it. The remaining material was then ground and extracted with water for three extractions, 50 pounds of water being used in all but one of the extractions where 40 pounds was used. After each extraction the udder material was placed in a cheese-cloth bag and allowed to drain for varying lengths of time. The udder material remaining after this treatment was then weighed. These weights are indicated in Table VII. A large part of this weight was water replacing the soluble materials washed from the udder. This remaining udder material was dried to constant weight and extracted with gasoline until all the fat was removed. It then was dried and ground and the percentage of remaining lactose determined. The determination of the percentage of water and fat removed from the remaining udder material is shown in Table VII.

Column 2 of Table VII shows the remaining udder material after it has been washed by the three extractions. It will be noticed that the amount of this material has a fairly close correlation to the yield of milk which the cows are giving.

The percentage of water and fat which was found in this material is shown in the third column. This water and fat varies between 85 and 91 per cent of the udder material. As would be expected from

the fact that the length of time in draining the udder was not constant for each cow, this percentage variation is quite random.

The weight of the remaining udder substance after the extraction of water and fat is shown in the fourth column of the table. The amount of udder substance varies between 1.4 and 2.3 pounds. On the basis of the total weight of the udder and its contained milk this dried material, gasoline- and water-soluble-free, is 5 to 10 per cent of the udder weight.

If the weight of the remaining udder substance is compared with the yield of milk which the cow is able to give, but little correlation is found. This lack of correlation is quite likely caused by the fact

TABLE VII.

Remaining Udder Material after Extraction, and Percentage of Water and Fat Removed from the Same.

Cow No	Remaining udder material	Percentage of water and fat	Weight of remaining substance	Milk yield total
111	15 7	87 7	1 93	20 3
124	16 9	89 6	1 76	27 9
132	14 5	87 3	1.84	25 9
136	16 5	86 1	2 29	29 5
148	15 5	90 8	1.43	21 8
154	15 2	89 3	1 63	17 7
97	12 0	84 7	1 84	6 8
114	11 0	87 4	1 39	6 9
153	14 5	89 6	1 51	17 8

that these cows were, in general, producers of about the same capacity. The lower productions of 97 and 114 represent the drying off of these cows. This insoluble udder substance may be regarded as largely supporting tissue for the secreting cells and therefore of a more or less permanent nature. By the method of treatment this material appears to be of about the same weight for the cows secreting milk and those which are approaching the dry period.

SUMMARY.

The results herein presented furnish exact critical evidence for the conclusion that the most of the milk is present as such in the udder of

dairy cattle at the time of milking. The amount of milk which may be secreted during milking cannot, on the basis of these results, be over 20 per cent of the milk yield of the cow.

The results show clearly that the size of the udder measures closely the amount of milk which the cow is able to secrete.

The results indicate that about 1/5 of a pound of secreting tissue is necessary for the secretion of a pound of milk during a period of 15 hours. The weight of the udder during the period that the cow is dry appears to be between 6 and 8 pounds.

[Reprinted from The Journal of Experimental Medicine, August 1, 1927, Vol. xlvi, No. 2, pp. 291–301.]

THE EFFECT OF HEAT ON ANTIBODIES.

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(Received for publication, May 4, 1927.)

Although it is well known that at certain temperatures antibodies are destroyed or inactivated, nevertheless with few exceptions detailed experiments covering this point are not readily available. T. Smith and Reagh (1) showed that there were two well defined types of agglutinin for the hog cholera bacillus; one they termed flagellar agglutinin, the other, body agglutinin. Beyer and Reagh (2) were able by a series of experiments to differentiate the flagellar and somatic (body) agglutinins by means of heat; the former was unimpaired when heated at 70°C. for 20 minutes, while the action of the somatic agglutinin was markedly impaired under these conditions. Joos (3) had previously called attention to the fact that when typhoid agglutinin was heated at 60–62°C. for 1 hour, a portion of the agglutinin was destroyed. More recently Orcutt (4) took up the study and showed that a temperature of 70°C. destroyed the somatic agglutinin to a considerable extent and 75°C. rendered it completely inactive. On the other hand, 70°C. failed to affect appreciably the flagellar agglutinin and 75°C. rendered it a little less active. It seems definite that certain types of agglutinin react differently to varying temperatures. The data in regard to the behavior of other types of agglutinins, precipitins, and hemolysins are not so definite.

In addition to a study of the specific effect of the various substances upon their respective antigens, it seemed of further interest to ascertain whether antibody was still capable of combining with its antigen although remaining in insufficient quantities to give visible reactions. It might also be possible that heat so affects some of the serum proteins that they no longer respond in a characteristic manner, thus rendering inoperative the usual physical phenomena used to interpret the results.

Complement fixation seemed to meet these objections. With these points in view, it was decided to study the effect of various temperatures on certain antibodies contained in the blood serum of rabbits.

EXPERIMENTAL.

Rabbits were immunized to various substances and when a sufficiently high titered serum was obtained the animals were bled and the serum collected and stored in the refrigerator. The serum containing agglutinin and hemolysin was

TABLE I.

The Effect of Various Temperatures on Flagellar Agglutinin.

	Dilutions of serum										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Unheated	C*	C	C	C	C	C	+++	++	+	+−	+−
Heated at °C											
65	C	C	C	C	C	++++	++	+	+	+−	−
70	C	C	C	C	+++	++	+	+	+	+−	−
75	+++	+++	++++	++++	+++	++	+	+	+−	−	−
80	+	+	+	+	+	+−	+−	−	−	−	−
85	+−	+−	+−	−	−	−	−	−	−	−	−
90	+−	+−	+−	−	−	−	−	−	−	−	−

* C indicates complete clumping of the antigen; ++++, marked agglutination without complete clumping of the test fluid; +++, well defined agglutination; ++, less well defined; +, definitely positive; +−, small deposits of clumped bacilli on the bottom of the tube.

diluted in four parts of normal NaCl solution and heated at various temperatures. The precipitin was diluted with equal parts of NaCl solution and then heated.

In all experiments corrected thermometers were used and the various materials heated in a deep water bath in tightly stoppered tubes for 20 minutes. The tests were always made with the same lot of specific antigen. When complement was employed it was of the same lot and of uniform concentration. The appended protocols afford examples of various observations.

Experiment 1.—Agglutinin was prepared by immunizing a rabbit with a motile strain of the hog cholera bacillus The serum was diluted with four parts of normal

salt solution and distributed in sterile tubes. One part was left unheated, the others were heated for 20 minutes at various temperatures. A portion of the contents of each tube was then tested for "flagellar" agglutinin with the motile strain. Another portion was tested with a non-motile strain of the hog cholera bacillus and a portion of the remainder used in the complement fixation tests further to confirm the findings. The protocols are submitted in Tables I and II.

These experiments substantiate the results of the previous workers. 75°C. for 20 minutes is a critical temperature at which the two agglutinins may be readily separated. The flagellar type resists temperatures considerably higher; even after exposure to 80°C. well de-

TABLE II.

The Effect of Heat on the Agglutinin for the Non-Motile Strain of the Hog Cholera Bacillus.

	Dilutions of serum								
	1 10	1 20	1·40	1 80	1 160	1 320	1 640	1·1,280	1.2,560
Unheated	C	C	C	C	+++	++	++	+	–
Heated for 20 min. at °C									
65	+	+	+	+	+	+	+	–	–
70	+	+	+	+	+	+–	–	–	–
75	–	–	–	–	–	–	–	–	–
80	–	–	–	–	–	–	–	–	–

fined agglutination occurs when the heated serum and antigen are mixed. At temperatures still higher it is possible to show that agglutinin still persists. It will be noted in Table I that in the lower dilutions the reaction has been interpreted as +–. If the small deposits found on the bottom and sides of the tube are examined microscopically it is found that they are composed of clumped masses of bacilli. If this experiment is repeated as a microscopic agglutination test, comparable results are obtained. The bacilli lose their motility and form small, loose clumps.

It might be argued that the antibody still remained in the serum, particularly in the case of the somatic agglutinin, but through some physical or other change in the globulin was incapable of reacting

with its antigen in the characteristic manner. As an additional control, to 10 volumes of the heated antiserum, 1 volume of fresh normal rabbit

TABLE III.

The Persistence of Antibody in Flagellar Agglutinin Exposed to Various Temperatures.

Treatment of agglutinin	Antigen	Complement	Antibody	Amboceptor	Hemolysis
Unheated	+	+	+	+	0*
	+	0	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	+−
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	+
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 85°C. for 20 min.	+	+	+	+	++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 90°C. for 20 min.	+	+	+	+	+++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C

* 0 indicates no hemolysis; C, complete; the plus signs, gradations from a very strong reaction (++++) to barely perceptible hemolysis (+−).

serum was added and the mixture tested. The addition of fresh normal rabbit serum failed to activate the inactivated antibody.

The resistance of the flagellar agglutinin to heat seemed so remarkable that a further control procedure seemed desirable. If it were

TABLE IV.

The Effect of Heat on Cow Serum Precipitin.

	Dilutions of antigen								
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
Unheated	+++*	+++	+++	+++	+++	+++	++	+	+−
Heated for 20 min. at									
°C									
60	+++	+++	++	++	++	++	+	+	+−
65	++	+	+	+	+	+	+	+−	−
70	+−	+−	+−	+−	+−	+−	+−	+−	−
75	−	−	−	−	−	−	−	−	−
80	−	−	−	−	−	−	−	−	−

* Precipitation has been recorded as follows: +++, the maximum; ++, less; +, weaker but well precipitated; +−, a trace of deposit.

TABLE V.

The Effect of Heat on the Complement-Binding Properties of Precipitin.

Treatment of precipitin	Antigen	Complement	Precipitin	Amboceptor	Hemolysis
Unheated	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	++++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	C
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C

TABLE VI.

The Effect of Heat on Hemolysin.

	Dilutions of hemolysin										
	1 10	1 20	1 40	1 80	1 160	1 320	1 640	1·1,280	1 2,560	1 5,120	1 10,240
Unheated	C	C	C	C	C	++++	++++	++++	++++	+++	++
Heated at °C											
65	C	C	C	C	++++	++	+	+	+-	-	-
70	C	C	C	C	+++	+-	-	-	-	-	-
75	C	++++	++	+-	-	-	-	-	-	-	-
80	+	-	-	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-

possible to show that sufficient antibody remained in heated serum to inhibit complement, the experiment would be further substantiated. With this in view, the whole series was tested, but as the sample exposed to 65°C. behaved like the unheated mixture the results are not given. The antigen consisted of actively motile hog cholera bacilli in normal NaCl solution. The complement was fresh guinea pig serum. The washed red cells of the sheep and the specific amboceptor were employed. These substances were always used in the same concentrations. The antiserum consisted of 0.02 cc. of the serum diluted 1:4 with salt solution. The results are given in Table III

TABLE VII.

The Effect of Heat on Red Cell Agglutinin.

	Dilutions of agglutinin					
	1 10	1 20	1 40	1 80	1 160	1 320
Unheated	+++*	+++	+++	+++	++	–
Heated at °C						
65	+++	+++	+++	+++	M+	–
70	+++	+++	+	M–	M–	M–
75	++	++	M–	M–	M–	M–
80	M–	M–	–	–	–	–
85	M–	M–	–	–	–	–

* Agglutination in this table has been recorded as follows: +++, maximum; ++, less strong; +, clumps definite to the naked eye; M +, the presence of microscopic clumps; M –, the absence of microscopic clumps.

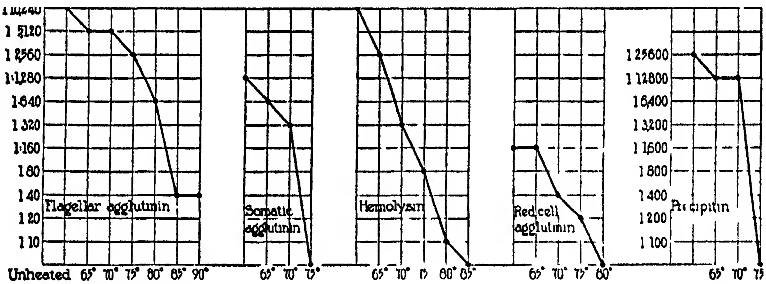
It is apparent from the results given in Table III that sufficient flagellar agglutinin, heated to 70°C. for 20 minutes, remains to divert completely the complement. As the temperature is increased less of the complement is diverted, although the inhibition is strong even after 80°C. for 20 minutes. Less of the complement is fixed when the serum is heated to 85°C. and 90°C., although it is apparent that sufficient antibody still remains to influence the intensity of the hemolysis. It can be said that the findings recorded in Table I are confirmed by the results of complement fixation.

It seemed of interest to test various other antibodies under the

same conditions. With this in view, a cow serum precipitin, and anti-sheep hemolysin and red cell agglutinin were subjected to the same method of procedure. In the instance of precipitin the serum was diluted with an equal part of NaCl solution, the hemolysin and hemagglutinin were diluted 1:4.

The effect of heat on precipitin and the persistence of sufficient antibody to bind complement are recorded in Tables IV and V.

It is obvious that the visibility of the precipitin reaction is destroyed when the precipitin is heated to 75°C. for 20 minutes. Complement fixation tests confirm this observation. It might be argued that the comparison between precipitin and the other antibodies is hardly a fair one since the serum mixtures were more concentrated in the



TEXT-FIG. 1. The effect of heat on the various antibodies. Logarithmic curves based on the data given in the tables.

former cases. Such is not the case, since precipitin diluted 1:4 and heated gave the same results in complement fixation tests as that diluted 1:1 and treated in the same manner.

The evidence of the effect of heat on hemolysis and red cell agglutinin is given in Tables VI and VII.

With the increase in temperature the hemolytic titer declines. 65°C. for 20 minutes has an appreciable effect, and increase to 75°C. materially affects the antibody, and only a trace remains after heating to 80°C. A similar result is obtained with the red cell agglutinin except that no agglutinin can be demonstrated in the serum held at 75°C. for 20 minutes.

DISCUSSION AND SUMMARY.

It is possible by means of curves to depict graphically the behavior of the various antibodies under various conditions. Logarithmic curves based on the data presented in the tables are submitted in Text-fig. 1.

In general it is evident that antibody destruction goes on gradually as the temperature is increased. Thus 65°C. for 20 minutes diminishes the activity of all the antibodies with the exception of red cell agglutinin, and in this case although the final titer was the same evidently some of the antibody was inactivated, since the reaction was weaker in the higher dilutions. It can, then, be said that 65°C. for 20 minutes appreciably affects the activity of all the antibodies tested. When the temperature is increased to 70°C. more marked differences are apparent. Here both types of the bacterial agglutinin and the precipitin are fairly stable when compared with hemolysin and red cell agglutinin. In both instances there is a sharp decline in the activity of the antibody. 75°C., however, is even a more critical temperature since at this point the somatic bacterial agglutinin and the precipitin are completely inactivated. The hemolysin and hemagglutinin behave alike. The flagellar agglutinin is the most resistant of the group to this temperature. When the temperature is increased to 80°C. the red cell agglutinin is completely inactivated, but sufficient hemolysin still remains to give a slight reaction at the lowest dilution. A further increase to 85°C. completely destroyed the hemolysin but left a definite amount of flagellar agglutinin; in fact, 90°C. for 20 minutes did not completely destroy this substance, since well defined clumps in the lower serum dilutions could be detected on microscopic examination. In this respect, then, the observations of Beyer and Reagh and Orcutt that there is a well defined difference between the two agglutinins for the hog cholera bacillus have been confirmed. However, each substance tested, with perhaps two exceptions, differs in its behavior to heat. It is of interest to point out the similarities in the reaction of somatic agglutinin and precipitin. Both are diminished when heated to 65°C.; 70°C. further affects the agglutinin, but not the precipitin; 75°C. completely inactivates both.

The assumption that the substances are apparently destroyed when they cease to react visibly with their respective antigens seems well founded since they cannot be reactivated with normal serum and no longer react to divert complement when combined in a hemolytic system.

It might be of interest to mention briefly other experiments in which the temperature was kept constant and the time varied. Thus temperatures of 50–55°C. and 60°C. maintained for 8 hours had no effect on antibody. 60°C. for 4 days failed to alter materially the flagellar agglutinin, although the same temperature for 24 hours inactivated the somatic agglutinin and the cow serum precipitin. Hemolysin deteriorates slowly at 60°C., so that after 4 days the serum, which originally reacted at a dilution of 1:10,240, only titered 1:160. The red cell agglutinin was about as resistant as the hemolysin in that a little still remained at the end of the test period. The experiments while incomplete add further proof that the somatic agglutinin and the precipitin are the least resistant to heat, while the flagellar agglutinin is on the whole comparatively stable.

A final experiment was performed to determine, if possible, at what temperature rabbit serum globulin was inactivated. With this in view, globulin was obtained by precipitation with ammonium sulfate, and a series of guinea pigs and chickens received several intraperitoneal injections. In no instance was a globulin precipitin obtained. By immunizing fowls in a similar manner with small quantities of rabbit serum good precipitin was obtained. The diluted rabbit sera heated at various temperatures for 20 minutes were tested for their antigenic activity with rabbit serum precipitin. It was found that diluted serum heated to 90°C. for 20 minutes reacted to about the same antigenic level as that not heated. Even boiling for 20 minutes failed to reduce greatly its antigenic properties. Paradoxically the visibility of the reaction was more intense with the antigen heated at the higher temperatures. The phenomenon was altogether so opposed to the usual conceptions of the inactivation of antigens that the subject will be gone into with more detail in a later communication. Although it is not possible to show definitely in the experiments that the globulin is or is not inactivated at certain temperatures, nevertheless it appears

probable that certain of the antibodies are destroyed at temperatures below that capable of greatly altering globulin.

It must be recognized that comparisons cannot be made between similar antibodies in the serum of different species, since somatic agglutinin in rabbit serum resisted 70°C. for 20 minutes, although the same agglutinin in cow serum was destroyed at 65°C.

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[Reprinted from The Journal of Experimental Medicine, August 1, 1927
Vol. xlvi, No. 2, pp. 303–314.]

AGGLUTINATION BY PRECIPITIN.

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(Received for publication, May 23, 1927.)

The similarity of the reactions of agglutination and precipitation has been commented on by many. Nicolle (1) combined a watery extract of the typhoid bacillus with other microorganisms or with finely divided particulate matter, and after the addition of a 1:10 concentration of antityphoid serum obtained agglutination of the bacteria or inert particles. Kraus and von Pirquet (2) observed precipitation when specific bacterial antiserum was added to extracts of the organism. Arkwright's (3) experiments were along similar lines. When he added a clear watery extract of the typhoid bacillus to a typhoid culture rendered inagglutinable by washing, and further added dilute acid or diluted antityphoid serum, agglutination resulted. The same effects were obtained when *B. coli* or other organisms or particulate matter were added to the typhoid bacillus extract and mixed with antityphoid serum. Thus he points out that the added bacteria or particulate matter in the presence of the specific extract act in a similar manner to *B. typhosus*. He considers the reactions of precipitation and agglutination analogous.

In the course of other experiments (4) diluted normal rabbit serum was heated at various temperatures and tested with a specific precipitin. On the whole weak reactions were obtained with unheated serum and with serum heated at temperatures up to 70°C. for 20 minutes. When the antigen was heated at 75°C. or higher, the reaction was intensified and at certain dilutions it resembled more nearly agglutination in intensity and amount of precipitate. The results were so striking that they seemed worthy of further investigation.

EXPERIMENTAL.

Throughout the experiments three precipitins were used. The first few observations were made with a precipitin prepared by injecting fowls with rabbit serum. Later experiments were made with cow serum precipitin and crystallized egg albumin antiserum obtained from rabbits injected with the respective antigens. The methods used are recorded with the experiments reported in detail in the following pages.

TABLE I.

Effect of Heating Rabbit Serum (Antigen) on Specific Precipitation.

Rabbit serum (antigen)	Dilutions of antigen						
	1 80	1 160	1 320	1 640	1 1,280	1 2,560	1 5,120
Unheated	+*	+	+	++	+	+	+
Heated for 20 min. at °C.							
75	−	−	+	+	++	++	+
80	−	−	+	++	++	++	+
85	−	−	+	+++	+++	+++	+
90	−	−	++	+++	+++	+++	+
Boiled for 20 min.	++++	++++	+++	+++	+++	++	+
Autoclaved, 14 lbs. pressure for 20 min.	+	+	+−	−	−	−	−

* The bulk of precipitate has been recorded as follows: ++++, an extremely heavy deposit; +++, a heavy deposit; ++, less bulky; +, a well defined precipitate; +−, a trace of granular deposit.

It was mentioned that by heating serum antigens the reactions were intensified on the addition of specific precipitin. Inasmuch as these experiments afforded the basis for the whole problem they will be given in detail.

Experiment 1.—Rabbit serum was diluted in the proportion of 1 to 4 with 0.9 per cent salt solution and heated at various temperatures for 20 minutes in tightly stoppered tubes in a deep water bath and tested with specific precipitin obtained by immunizing a fowl. Inasmuch as the antigen heated at 60–65° and 70°C. behaved like the unheated serum the results are not given in the table. In the tests 0.1 cc. of the precipitin was added to each tube of diluted antigen All tubes were incubated for 2 hours and refrigerated overnight. The amount of precipitation is recorded in Table I.

The result was on the whole so unexpected that the experiment was repeated, with similar results. Another experiment in which cow serum antigen was heated and then tested with its specific precipitin yielded similar results. Even boiling the diluted cow serum antigen for 20 minutes served to increase the bulk of the precipitate in the lower dilutions.

It might be argued that the antigenic protein was denatured as the result of heating and that it would react with any foreign serum. To test this possibility the heated rabbit serum was treated with normal fowl serum and the heated cow serum with normal rabbit serum. No reaction occurred in the rabbit-fowl serum series except with the autoclaved antigen, where there was a little precipitate in the lowest dilution. The cow serum boiled for 20 minutes also gave a slight reaction at the lowest dilution when mixed with normal rabbit serum. The results given in Table I must be considered specific.

It was possible to correlate to a certain extent the intensity of the precipitation with the degree of coagulation evidenced by the turbidity of the serum. When rabbit serum is diluted with salt solution and heated at 70°C. it is not greatly altered in appearance. As the temperature is increased the turbidity becomes more marked so that the mixture heated at 85°C. or higher is nearly opaque.

For the purpose of interpreting the results it may be assumed that portions of the diluted antigenic serum coagulate during heating. The mixture then contains in a liquid state unaltered antigen and suspended particles of coagulated protein. It may be further assumed that the coagulated particles are covered with active antigen and when brought in contact with a specific serum may be likened to a bacterial suspension to which specific agglutinin is added. In the first case an agglutination of the protein particles would result when precipitin was added, and in the other bacterial agglutination would result on the addition of agglutinin. The work of many tends to support this hypothesis.

Coulter (5) showed that red cells agglutinate at pH 4.75, but when sensitized with serum the agglutination point was shifted to that of globulin (pH 5.3). Northrop and De Kruif (6) found that a mixture of bacteria and egg albumin or bacteria and globulin behaved toward acid like solutions of the respective proteins; the isoelectric point of the organism shifted to that of the added protein. Loeb (7)

TABLE II.

Effect of the Addition of Bacteria or Particulate Matter to Antigen.

	Dilutions of cow serum								
	1 1,280	1 2,560	1 5,120	1 10,240	1 20,480	1 40,960	1 81,920	1 163,840	1.327,680
Cow serum + *B. abortus* + cow serum precipitin	++++	++++	++++	+++	+++	++	+−		
Cow serum + cow serum precipitin ..	+++	+++	+++	++	+	+	−		
Cow serum + *B. abortus* + normal rabbit serum	−	−	−	−	−	−	−		
Cow serum + collodion particles + cow serum precipitin....	++++	++++	+++	+++	+++	++	++	++	+
Cow serum + cow serum precipitin	++++	+++	+++	+++	+++	+	+	+−	−
Cow serum + collodion particles + normal rabbit serum.......	−	−	−	−	−	−	−	−	−

has shown that collodion particles treated with proteins acquire a film of protein on their surfaces. This film causes the particles to assume the character of protein particles in their cataphoretic behavior.

In order to substantiate further the hypothesis that coagulated particles of serum protein are agglutinated on the addition of specific precipitin a further series of experiments was performed.

Experiment 2.—If the coagulated particles of protein in the heated serum act as more or less inert material covered with antigen, then the addition to antigen of inert material, such as bacteria or particulate matter, should increase the intensity of the reaction when a specific precipitin is added to the suspension. *B. abortus* was suspended in 0.9 per cent NaCl solution and the turbidity adjusted to 3.5 by the Gates apparatus. This suspension was used as the fluid in which the cow serum was diluted. 0.1 cc. of cow antiserum was added to each tube. The results are given in Table II. A similar observation in which sufficient collodion particles were added to salt solution to make a faintly turbid suspension which was used to dilute the antigen is included in the table. In both series adequate controls containing cow serum and bacteria or collodion particles were tested with normal rabbit serum For comparison the results of the usual precipitation tests are included.

It is evident from Experiment 2 that the addition of bacteria or particulate matter increases the intensity of the reaction in a manner similar to that observed in Experiment 1 where the antigen was heated sufficiently to cause turbidity. It is of further interest to note that the addition of bacteria or collodion particles produced reactions at higher dilutions. Microscopic examination of the sediment revealed definite clumping of the bacteria and collodion particles.

It will be noted in the experiments thus far that the tests have been conducted as precipitin tests, the antigen has been diluted but the antibody kept constant. It might be argued that, as Arkwright has contended, during the union of antigen and antibody a web is formed and that the bacteria or collodion particles are enmeshed and fall to the bottom of the tube. To show that this is not the proper interpretation of the phenomenon several experiments were performed which conform more closely to the procedure usually employed in bacterial agglutination.

Experiment 3.—0.25 cc. of a suspension of collodion particles was added to 5 cc. of normal cow serum. The mixture was incubated for 3 hours and then refriger-

ated overnight. The next day the liquid was drawn off and mixed with an equal volume of normal salt solution. It was centrifuged at high speed and the sediment resuspended in NaCl solution and again centrifuged. The process of washing was repeated twice more. The particles were then suspended in salt solution and tested with cow serum precipitin. Some of the third wash fluid was retained and tested for the presence of cow serum. The results are given in Table III.

If a solution of crystallized egg albumin is substituted for the cow serum and the particles washed three times, resuspended in NaCl solution, and various dilutions of crystallized egg albumin antiserum added, a similar result is obtained, as is shown in Experiment 4.

TABLE III.

Agglutination of Collodion Particles Sensitized with Cow Serum by Cow Serum Precipitin.

	Tested with	Amount of test material, in cc					
		0 1	0 2	0 01	0 002	0 001	0 0005
Collodion particles sensitized with cow serum and washed	Cow serum precipitin	C*	C	C	+++	+	–
	Normal rabbit serum	–	–	–	–	–	–

	1 0 cc	0 5 cc.	0 1 cc
The last wash fluid tested with 0.1 cc. cow serum precipitin.	+–	–	–

* C indicates complete agglutination; +++, a strong agglutination; and +, a slight agglutination.

Experiment 4.—0.4 cc. of a suspension of collodion particles was added to 5 cc. of a 2.5 per cent solution of crystallized egg albumin. After 1 hour's incubation, 5 cc. of normal salt solution was added and the whole reincubated for 30 minutes. The mixture was then centrifuged for a brief interval and the supernatant fluid withdrawn and centrifuged rapidly. The supernatant fluid was discarded and the sediment resuspended in salt solution containing a small quantity of alkali (0.2 cc. N/20 NaOH to 10 cc. NaCl solution). The centrifuging and washing were repeated twice more. The particles were then suspended in slightly alkaline salt solution and tested with crystallized egg albumin antiserum. The results are given in Table IV.

It is evident that the collodion particles attach to themselves sufficient protein, as Loeb maintained, to react in a specific manner

in the presence of specific precipitin. Hitchcock (8) has been able to show that egg albumin adheres to collodion membranes in amounts sufficient to be detected quantitatively.

If it were possible to show that bacteria on coming in contact with proteins retained a film of the antigenic substance which caused them to agglutinate on the addition of a specific precipitin, then the evidence that precipitin and agglutinin were identical would be complete. With this in view a number of experiments were performed. Inasmuch as several species of bacteria react to certain proteins in

TABLE IV.

Agglutination of Collodion Particles Sensitized with Crystallized Egg Albumin by Crystallized Egg Albumin Precipitin.

	Tested with	Amount of test material, in cc					
		0.05	0.02	0.01	0.005	0.002	0.001
Collodion particles sensitized with crystallized egg albumin and then washed 3 times	Crystallized egg albumin precipitin	C	C	C	C	+++	+
	Normal rabbit serum	–	–	–	–	–	–

	1.0 cc	0.5 cc	0.1 cc
The last wash fluid tested for the presence of crystallized egg albumin with 0.1 cc. of precipitin . . .	–	–	–

a relatively uniform manner, only a single experiment will be reported in detail.

Experiment 5.—The growth from a 24 hour agar slant culture of a non-motile strain of the hog cholera bacillus was suspended in 3.5 cc. of normal cow serum which had been previously heated to 65°C. It was then incubated for 3½ hours and an excess of salt solution added and the whole mixed. The mixture was centrifuged rapidly and the supernatant liquid replaced with salt solution. The centrifugation and washing were repeated twice and the bacteria suspended in NaCl solution and tested with cow antiserum. Some of the final wash fluid was retained and likewise tested for cow serum. As a control the same amount of culture was suspended in salt solution, washed twice, and tested with the cow serum precipitin. The results of a typical experiment are recorded in Table V.

It is apparent from the table that a portion of normal cow serum adheres to the bacteria in sufficient quantity to give a characteristic agglutination when mixed with the precipitin. The experiment was repeated with different organisms and comparable results were always obtained. The best results were obtained when the cow serum heated to 65°C. for 20 minutes was used for sensitization. When unheated cow serum is used, the results are about the same; it however usually clumps the bacilli so that aggregates are dealt with and the results for this reason are open to criticism. If serum is diluted 1:5 and used to sensitize the organisms, the reactions are less intense although agglutination occurs on the addition of the precipitin.

TABLE V.

Agglutination of Bacteria Sensitized with Cow Serum by Cow Serum Precipitin.

	Cc of cow antiserum					
	0 02	0 01	0.005	0.002	0 001	Control
Bacteria sensitized with cow serum and subsequently washed twice..	C	++++	++	+	+−	−
Unsensitized bacteria washed in NaCl solution	++	−	−	−	−	−

	1 0 cc	0 5 cc
Last wash solution tested with 0.1 cc. precipitin for cow serum	−	−

Thus far it has not been possible to sensitize bacteria with various concentrations of crystallized egg albumin. It is true that specific precipitin added to a mixture of bacteria and crystallized egg albumin will agglutinate the bacteria, nevertheless when bacteria are soaked in crystallized egg albumin and subsequently washed they are not agglutinated by the egg albumin antiserum. This experiment was varied in respect to the concentration of egg albumin, pH concentration of the egg albumin, temperature, and time, but in no instance could agglutination with precipitin be obtained after the bacteria had been washed.

DISCUSSION.

The experiments reported strengthen the belief that the intensity of the reaction when precipitin is added to heated serum antigen is increased because coagulated serum proteins in suspension are covered with undenatured antigen, which under the conditions are agglutinated. The visibility of the reaction is enhanced because of the greatly increased flocculation.

The experiments are of interest in other respects. First the evidence that precipitin and agglutinin are similar is strengthened. When precipitin and its specific antigen are mixed turbidity occurs, later the flocculi increase to the point of visibility and are precipitated. The same reaction can be obtained by mixing bacteria or inert particles with antigen, then adding the precipitin. Here the particles are made up of clumps of bacteria or the inert particles and presumably antigen and antibody. It might be argued that a web similar to that inferred by Arkwright in agglutination was formed in the antigen-antibody union, and that the bacteria were enmeshed in the course of this flocculation. However, if collodion particles were mixed with cow serum or crystallized egg albumin and then washed until free antigen no longer remained in the wash solution, they behaved like bacteria sensitized to cow serum and subsequently washed. The addition of the specific precipitin in increasing dilutions produced agglutination of the bacteria or inert particles. In the experiments of other workers the evidence is presumptive; however, since the antisera employed contained both bacterial agglutinin and precipitin, the presence of both substances complicated the problem. In the experiments here reported a precipitin free of the bacterial agglutinin is shown to behave like bacterial agglutinin.

In a previous communication(4) it was shown that somatic bacterial agglutinin and cow serum precipitin behave in a similar manner toward heat; both are destroyed at 75°C. and both fail to resist 60°C. for 24 hours, although flagellar agglutinin, hemolysin, and hemagglutinin resist these temperatures.

It is of further interest to comment on the behavior of certain proteins under the experimental conditions. If collodion particles are exposed to crystallized egg albumin or cow serum, there occurs a

firm union between the particle and the antigen. Loeb has described this as the deposition of a protein film. He suggested that protein denaturation probably accounted for the deposition of the film. How much denaturation takes place is a question, since in a preliminary report Wu, TenBroeck, and Li (9) state that denatured egg albumin, whatever the agent of denaturation, is immunologically different from egg albumin. The behavior of bacteria toward the two types of proteins is sharply contrasted. There is a definite fixation of the proteins of cow serum to the bacterial cell sufficient to withstand three washings with salt solution. On the other hand, this is not true with crystallized egg albumin. Union evidently occurs, as shown by Northrop and De Kruif (6), but either the albumin is removed by salt solution or so denatured by the bacterial cell that sufficient original protein no longer remains to react when specific precipitin is added. Confirmatory evidence was obtained by means of acid agglutination in that the bacteria soaked in cow serum and then washed agglutinated at about the same acid concentration as a mixture of cow serum and bacteria. Such was not the case when a mixture of crystallized egg albumin and bacteria, and bacteria soaked in the albumin solution and subsequently washed were tested with various concentrations of H ions.

A further series of experiments not reported suggests that precipitin may under certain conditions act as opsonin. If bacteria, antigen, and precipitin are mixed and incubated for 1 hour, then normal rabbit serum and washed rabbit leucocytes added and permitted to act for an hour or more, in the tubes containing bacteria, antigen, and precipitin about three times as many of the leucocytes are found to have taken up the organisms as is the case in the tubes which contain only bacteria plus normal rabbit serum and precipitin or normal rabbit serum and antigen. The difference in the number of organisms per cell is very large; where the immunological series is complete the cells are packed with bacteria, while in the others relatively few organisms are taken up. The results are not so striking as in experiments in which a strongly reacting agglutinin was employed, nevertheless sufficient agglutination and opsonization take place to prepare the bacterial cells for phagocytosis.

It is of interest to note that the experiments tend to corroborate the conception of Avery and Heidelberger (10) that agglutination is a cell surface phenomenon. They point out that the nature of the substance at the periphery of the bacterial cell may determine the readiness of response and even the specificity of the reaction. This seems to be the case when inert particles are coated with crystallized egg albumin or cow serum or bacteria coated with cow serum; the added protein adheres to the particles or bacterial cells and on the addition of the specific precipitin they behave like bacteria in the presence of their specific agglutinin.

SUMMARY.

Serum (antigen) when heated at a temperature sufficient to cause definite clouding reacts more intensely with a specific precipitin than a portion of the unheated serum or samples heated at lower temperatures. The phenomenon is explained on the basis that coagulated protein in suspension is covered with undenatured antigen and the addition of precipitin causes agglutination of the coagulated protein. Similar phenomena are obtained when bacteria or collodion particles are mixed with diluted serum (antigen) and precipitin added; the particles or bacteria agglutinate and increase the visibility of the reaction.

Further, it is shown that collodion particles sensitized with cow serum or crystallized egg albumin and subsequently washed until the washing fluid no longer contains the antigenic substance will agglutinate when small quantities of specific precipitin are added. Bacteria sensitized with cow serum and subsequently washed until cow serum no longer remains in the washing solution agglutinate when cow antiserum at fairly low concentration is added. It was not possible to show that bacteria soaked in crystallized egg albumin and subsequently washed retained on their surfaces sufficient undenatured egg albumin to react to crystallized egg albumin precipitin.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1927, Vol xlv, No. 6, pp. 1037–1044.]

BACTERIOPHAGE ISOLATED FROM THE COMMON HOUSE FLY (MUSCA DOMESTICA).

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(Received for publication, March 7, 1927.)

INTRODUCTION.

It is known, through the work of others, that the house fly, when fed cultures of various pathogenic bacteria, harbors them for a longer or shorter time but that they finally disappear entirely. Various explanations of this elimination have been offered or are suggested by the facts presented. Wollman (1) regarded the process as entirely mechanical. In the case presented by Jones and Little (2) an actual rapid sterilization was observed in which more subtle factors are obviously concerned.

Glaser and Sanderson, in unpublished observations from this laboratory, found in house flies bacteriophage active against *Staphylococcus muscæ*, a microorganism which gives rise to a definite disease of this insect (Glaser (3)). This observation offered the suggestion that bacteriophage might be found more widely active and might be concerned in the natural process of removal of foreign bacteria when these are fed to the fly. Duncan (4) has recently studied the same problem and discovered a bactericidal principle active against many species of bacteria in the gastrointestinal tracts of various insects, the fly among them. The references above generally commented on are more specifically as follows.

Graham-Smith (5) found that, with flies fed on cultures, *B. prodigiosus* (page 96) could survive in the crop only about 17 days. *B. enteritidis* Gärtner (page 146) could be recovered from the gut contents up to the 7th day. *B. typhosus* (page 130) up to the 6th day, and *V. choleræ* (page 173) for 2 days after ingestion by the insect. Manson-Bahr (6) found that *B. dysenteriæ* Shiga, from a culture, survived in the gut contents of flies 4 days. Naturally infected flies, he thought, carried the organ-

isms for a much longer time. Wollman (1) observed that flies fed on cultures of *B. typhosus* and *B. dysenteriæ* and then transferred daily to aseptic surroundings became free of the specific bacteria in from 8 to 10 days. Jones and Little (2), in conducting an investigation on infectious ophthalmia of cattle, made the observation that the causative diplobacillus was not capable of surviving as long as 5 minutes in the gastrointestinal tract of the house fly. Duncan (4) isolated a bactericidal principle from the alimentary tracts of a number of insects and arachnids. He included *Musca domestica* in the series of insects found by him to contain this bactericidal principle in their gastrointestinal contents. He considered that this principle might be a bacteriophage but was forced to discard the possibility as the substance he described exhibited none of the essential properties of this principle.

The experimental work recorded in the following pages was conducted with the purpose of determining any bactericidal action which might pertain to the house fly, and its result has been to identify bacteriophage very active against a number of bacteria, as well as an inhibitory substance, not identified with bacteriophage, and active still more widely.

EXPERIMENTAL.

1000 house flies were obtained from the vicinity of a hog lot, etherized and ground in a mortar with physiological salt solution in the proportion of 10 flies per cc. This mixture was filtered first through paper, then through a Berkefeld N filter. A clear, dark, straw-colored fluid was obtained which gave no bacterial growth when incubated on the ordinary culture media This fluid was kept at refrigerator temperature and was used as the starting point for most of the experimental work here reported.

Two organisms were used at the outset to determine whether or not the fly filtrate just described contained substances which were either bactericidal or capable of inhibiting bacterial growth. One of these was a non-mucoid strain of *B. coli* 223 of calf origin (7), and the other *B. paratyphi* Type I of guinea pig origin (8)

Plain bouillon (pH 7.5), 5 cc per tube, was used as the culture medium and the fly filtrate was added to the bouillon before inoculation. 1, 0.5, 0.25, and 0.1 cc. amounts of fly filtrate were added to four separate tubes in each series and one tube in each was kept as a control. One loopful of a 24 hour bouillon culture was used in making each inoculation. The tubes were read at 6 and 24 hours with the results given in Table I.

This experiment made it evident that the fly filtrate contained some substance which was inhibitory or bactericidal for *B. coli* (calf) but

which exerted no such action upon *B. paratyphi* Type I (guinea pig). This apparent specificity suggested the possibility that the factor might be bacteriophage. Each tube of the two sets of cultures was therefore passed through a Berkefeld N filter and the experiments were

TABLE I.

	B paratyphi Type I (guinea pig)		*B coli* (calf)	
	6 hrs	24 hrs.	6 hrs.	24 hrs
cc.				
1	Moderately turbid	Turbid	Clear	Clear
0.5	" "	"	"	"
0.25	" "	"	"	"
0.1	" "	"	"	"
Control	" "	"	Moderately turbid	Turbid

TABLE II.

Dilution	*B paratyphi* Type I (guinea pig)			*B. coli* (calf)		
	6 hrs.	24 hrs	48 hrs	6 hrs	24 hrs	48 hrs
10^{-1}	C.*	M.T.	M.T.	C.	V.S.T.	S.T.; agglutinated
10^{-2}	"	"	"	"	"	" "
10^{-3}	"	"	"	"	"	" "
10^{-4}	"	"	"	"	S.T.; agglutinated	M.T., "
10^{-5}	S.T.	S.T.	S.T.	"	" "	" "
10^{-6}	"	"	"	"	" "	" "
10^{-7}	M.T.	"	"	"	" "	" "
10^{-8}	"	"	"	S.T.	" "	" "
10^{-9}	"	"	"	"	" "	" "
Control	"	T.	T.	M.T.	T.	T.

* C. = clear; V.S.T., very slightly turbid; S.T., slightly turbid; M.T., moderately turbid; T., turbid.

repeated using the bouillon filtrate. The dilutions were made this time from tube to tube increasing by successive powers of ten. A single pipette was used for each series. The results are given in Table II.

After 48 hours incubation, each tube of these two sets of cultures was passed through a Berkefeld N filter and the experiment repeated with results similar to the above.

TABLE III.

Dilution	*B. typhosus* (Rawlings)			*B paratyphi* Type I (guinea pig)			*B coli* (calf)			*Staphylococcus muscæ*		
	6 hrs.	24 hrs.	48 hrs	6 hrs	24 hrs.	48 hrs.	6 hrs.	24 hrs	48 hrs	6 hrs.	24 hrs.	48 hrs.
10^{-1}	C.*	C.; Sed.	C.; Sed.	C.	M.T.	M.T.	C.	C.	S.T.	C.	C.	C.
10^{-2}	"	" "	" "	"	"	"	"	"	"	"	"	"
10^{-3}	"	" "	" "	S.T.	"	S.T.	"	S.T.	"	"	"	"
10^{-4}	"	" "	" "	"	"	"	"	"	"	"	"	"
10^{-5}	"	" "	" "	T.	"	M.T.	"	"	"	"	"	"
10^{-6}	"	" "	" "	"	T.	"	"	"	"	"	"	"
10^{-7}	"	" "	" "	"	"	"	S.T.	"	"	"	"	"
10^{-8}	"	S.T.; "	S.T.; "	"	"	"	"	"	"	S.T.	"	"
10^{-9}	S.T.	T.	T.	"	"	T.	M.T.	M.T.	"	"	"	"
10^{-10}	"	"	"	"	"	"	"	T.	T.	"	M.T.	M.T.
10^{-11}	M.T.	"	"	"	"	"	"	"	"	"	T.	T.
10^{-12}	"	"	"	"	"	"	"	"	"	"	"	"
Control	"	"	"	"	"	"	"	"	"	"	"	"

* C. = clear; S.T., slightly turbid; M T., moderately turbid, T., turbid; Sed , sedimentary growth.

Plates were next made and these showed typical plaque formation in both series. Individual colonies on solid media showed notched and stellate forms. The inhibition of growth in series by dilutions of the filtrates and the formation of plaques definitely characterizes the action as that of bacteriophage.

The activity of the original fly filtrate against a wider range of bacteria was then tested. Preliminary determinations were carried out using a single pipette for each set of dilutions, while the experiments to determine the definite end-points of activity, those given in Tables III and IV, were done using a fresh pipette for each dilution.

TABLE IV.

Organism	Highest dilution showing inhibition of growth at 6 hrs
Staphylococcus aureus	1:1000 (complete)
Streptococcus C 54	1:100 "
" 744	1:10 (complete). 1:10,000 (partial)
" C 55	1:10,000 (partial)
Pneumococcus I	1:10 (complete). 1:10,000 (partial)
" II	1:10,000 (complete)
Bacillus of swine plague	1:1000 (partial)
Vibrio choleræ	No inhibition of growth
Friedländer's bacillus	" " " "
Bacillus proteus	" " " "

Whenever evidence of inhibition of growth was obtained filtrates were made and tested in order to develop any potential transmission in series. Inhibition, displayed in successive filtrates, was thus uncovered in the case of *B. typhosus* (Rawlings) and *Staphylococcus muscæ* in addition to the two species previously considered. The complete record, as finally determined for the four species, is shown in Table III.

The original filtrate inhibited the growth of certain other species in various dilutions but filtrates in these cases had no inhibitory action. The end-point of activity for these bacteria and the species for which no inhibitory action could be demonstrated are contained in Table IV.

The bacteriophage isolated was very active against *Staphylococcus muscæ*, giving complete inhibition of growth to a dilution of 10^{-9}. The lysis of this organism was complete and permanent and no secondary growth ever occurred in the tubes that were completely lysed at 48

hours. It was fairly active against *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-8}, but in the case of this organism all lysed tubes showed some secondary growth in the form of a sediment. Against *B. coli* (calf) it was also fairly active, giving evidence of lysis in a dilution of 10^{-9}. With this organism a secondary growth occurred in 24 hours also. The lytic principle active against *B. paratyphi* Type I (guinea pig) was a relatively weak one and a secondary growth always occurred. Lysis in this case seemed to progress for at least 48 hours but complete clearing of the cultures never resulted.

The nature of the inhibitory action which differs so definitely from bacteriophage is of considerable interest. One possibility seemed to be that it might be related as a precursor or "building stone" for the bacteriophage. To test this, one of the species susceptible to inhibition, but for which bacteriophage was not developed, was fed to flies for a period. These flies were extracted as before and the filtered extract was tested for the characteristic transmissible lysis for the bacterium fed. The result was negative. The detailed experiment follows.

Streptococcus C 55, a non-hemolytic strain originating in bovine mastitis (9), which was inhibited but not susceptible to lysis by the bacteriophage in the original fly filtrate, was used. About 200 flies were placed in a large glass jar and fed bouillon cultures of Streptococcus C 55 daily for 8 days At the end of this time the flies were etherized and ground in a mortar with physiological salt solution using 5 flies per cc. This material was passed through a Berkefeld N filter for sterilization and, from the filtrate, attempts were made to obtain bacteriophage capable of lysing cultures of Streptococcus C 55 These attempts were all unsuccessful and no bacteriophage active against this organism could be obtained.

DISCUSSION.

Physiological salt solution extracts of the house fly present bacteriolytic or inhibitory phenomena of two types which may have a bearing on the inability of certain pathogenic microorganisms to exist for more than a short period of time in the gastrointestinal tract of the insect. Bacteriophage active against at least four species of bacteria was found in a salt solution extract of flies; and another substance, growth-inhibiting, but not showing the essential characteristics of bacteriophage, was also present. This was active against four additional species.

In so far as relates to the bacteriophage, it is very likely that the fly filtrate contains a mixture of lytic principles, rather than a single bacteriophage capable of causing lysis of the four species. Thus neither the broth filtrate active against *B. coli* (calf) nor the one active against *B. paratyphi* Type I (guinea pig) had any lytic action on *Staphylococcus muscæ*. The filtrate active against *B. paratyphi* Type I (guinea pig) was also strongly lytic for *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) was somewhat active for *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-4}. The filtrate active against *Staphylococcus muscæ* failed completely to cause lysis of *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) caused no lysis of *B. paratyphi* Type I (guinea pig). No effort was made to adapt a lytic principle, active against one organism, to another of the group.

Because of the possibility that the non-bacteriophagic growth-inhibiting substance might be a precursor to true bacteriophage, the feeding experiment described was planned. No bacteriophage against the streptococcus fed could be obtained in this way. It is very likely that this non-bacteriophagic growth-inhibiting factor is the same as that observed by Duncan (4).

Within the range of the experimental observations, the lytic principle present in the fly filtrate may have been obtained either from the external parts of the fly or from its digestive tract. A much more elaborate technical procedure would be required to make this discrimination.

SUMMARY AND CONCLUSIONS.

1. Bacteriophage active against four species of bacteria was found in a salt solution extract of house flies.

2. A growth-inhibiting principle, not bacteriophage, active against four other species of bacteria was found to be present in the same extract.

3. An attempt to secure streptococcus bacteriophage by feeding to flies a streptococcus susceptible to the inhibitor but not to the bacteriophage of this filtrate was unsuccessful, indicating that the two activities are quite unrelated.

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